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REVIEW

PHOSPHATIDYLSERINE, ANOTHER PLAYER IN MACROPHAGE RECRUITMENT IN WHITE ADIPOSE TISSUE?

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Abstract

Macrophage infiltration in white adipose tissue (WAT) underlies the development of the obesity-associated chronic inflammatory state. In this article current knowledge is reviewed with respect to adipocyte-driven mechanisms responsible for macrophage recruitment and activation in WAT. Adipocyte hypertrophy, adipocyte hypoxia, altered adipokine profiles under stress conditions and adipocyte death is discussed. In addition, new data is provided that shows phosphatidylserine exposure in human adipocytes under hypoxic conditions. This may represent an additional mechanism that plays a role in macrophage recruitment in WAT.

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Introduction

Obesity is characterized by a chronic, low-grade inflammatory state of the adipose tissue. This inflammatory state is most likely initiated by changes in the adipocyte phenotype and fuelled by infiltrating macrophages producing pro-inflammatory cytokines and reactive oxygen species. The development of the inflammatory state in white adipose tissue (WAT) is considered an event that underlies the development of metabolic complications during obesity. To become inflammatory in the WAT, macrophages need to be recruited and activated. Adipocyte hypertrophy, changed adipokine profiles and adipocyte death are important events related to macrophage recruitment and activation. However, the mechanism(s) for macrophage-adipocyte interaction in WAT are still not completely resolved. Macrophages are predominantly found in hypoxic areas in WAT and form so-called crown-like structures around dead adipocytes. Surface exposed phosphatidylserine (PS), resulting from a collapse of membrane lipid asymmetry during cell death, provides

a recognition signal for phagocytic macrophages. In this review an update is provided on adipocyte-derived mechanisms for macrophage infiltration and activation. In addition, preliminary data is presented showing PS exposure in adipocytes under hypoxic stress, which may act as a mechanism for adipocyte-macrophage interactions.

Macrophages in WAT

Macrophages are detected in WAT of rodents and humans (reviewed in (1) and their numbers are positively correlated with WAT mass. Increased numbers are found in WAT of genetically obese mice (2,3) and diet-induced obese mice (3,4) and in subcutaneous (2) and visceral WAT (5) of obese subjects. In addition, a reduction of the number of macrophages in subcutaneous WAT was observed after surgery-induced weight loss (6). These results indicate a dynamic relationship between WAT mass and macrophage infiltration.

Two types of macrophages have been described in WAT: the 'resident' M2 macrophages which appear involved in adipogenesis and the M1 type macrophages which are denoted as infiltrating cells (7). Infiltrated macrophages contribute to the chronic low-grade inflammatory state found in adipose tissue (2,3,8) and are considered to progress insulin resistance (9). With respect to the presence of macrophages in the different WAT depots, an increased number of macrophages was found in visceral fat compared to subcutaneous fat both in mouse models (2,10,11) and human (12,13). This suggested a more prominent role for visceral fat in relation to the onset of insulin resistance. However, recently, pro-inflammatory macrophages were detected in subcutaneous fat, where the density of crown structures was higher in obese woman with metabolic syndrome (ObMS) compared to 'normal' obese woman (Ob) (14). In addition, the crown density was higher in subcutaneous fat compared to visceral fat in ObMS. Although these results seem contradictive to the idea that visceral WAT is primarily responsible for induction of insulin resistance (15), they are in line with a another study showing that subcutaneous fat, but not visceral fat, was correlated with insulin resistance in lean and obese men (16). In addition, it was shown before that crown-like structure density in subcutaneous WAT of obese woman correlated with insulin resistance (17). Furthermore, the results are consistent with a greater size of subcutaneous adipocytes compared to visceral adipocytes and support the view that visceral obesity is a consequence rather than a cause for insulin resistance (18). Apparently, macrophage infiltration occurs in both types of human WAT and both contribute to development of insulin resistance. Notably, adipocyte size seems an important factor in macrophage infiltration.

Recruited macrophages during diet-induced obesity have increased inflammatory properties in contrast to macrophages already present in adipose tissue (19). In addition, it appeared that non-adipose cells in human adipose tissue are responsible for the majority of inflammatory factors secreted by the adipose tissue (20), which can be reversed by weight loss (21). Together, this indicates that novel recruited macrophages rather than adipose-residing macrophages are involved in the obesity-induced pro-inflammatory state and may initiate insulin resistance.

Adipose tissue hypoxia and inflammation

Macrophages have been detected in hypoxic areas in mouse adipose tissue, together with cytotoxic T-cells (4). Although hypoxic areas have been observed in WAT from obese mouse and humans (22-24), presence of macrophages in hypoxia areas in human WAT has not been demonstrated yet. However, as will be discussed later, macrophages in human WAT surround necrotic adipocytes (25). Since the adipose tissue is highly innervated (26), the idea raised that the extensive expansion of adipocytes during pre-adipocyte hypertrophy may cause a diminished blood supply by restricted capillaries. Alternatively, due to expansion of the adipocyte cell volume the O_2 diffusion distances within the tissue may be reduced (27). Both can lead to hypoxic conditions. The hypoxia hypothesis was proposed first by Trayhurn and Wood when they suggested that hypoxia could play a role in adipose-tissue inflammatory processes (28). It is known that human pre-adipocytes subjected to hypoxic conditions are inhibited in their differentiation process (27). In addition, mature mouse and human adipocytes exposed to hypoxia show an adipokine deregulation with increased expression of pro-angiogenic and pro-inflammatory factors (29-31). Furthermore, we showed that hypoxia induces lipolysis in human adipocytes (Rosenow et al., in preparation). Increased release of free fatty acids from adipocytes may trigger pro-inflammatory responses in M1 macrophages by targeting Toll-like receptor 4 (TLR4) (32).

The key regulator for transmission the hypoxic response in adipocytes is hypoxia-induced factor 1a (HIF-1a) (33). Stabilization of HIF-1a either by chemically induced hypoxia or by low oxygen tension showed increased expression and secretion of inflammation-related adipokines such as interleukin-6 (IL-6), macrophage inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) with a concomitant decreased secretion of the insulin sensitizer adiponectin (33). Currently, our group performs a proteomics study on the effect of hypoxia on adipokine profiles from human adipocytes (Rosenow et al., in preparation). In a transgenic mouse model, constitutively expressing HIF-1a, the WAT showed fibrotic lesions with associated insulin resistance (34). Together, it is increasingly recognized

that hypoxia in WAT plays an important role in changing the adipocyte phenotype, which may contribute to recruitment and activation of macrophages.

Adipokines and macrophage infiltration

The adipogenic process comprises pre-adipocyte hypertrophy and proliferation of (novel recruited) pre-adipocytes (35,36). However, in T2D patients it is shown that expression of genes involved in pre-adipocyte proliferation was decreased (37). This suggests that impaired adipogenesis, which leads to hypertrophy of existing pre-adipocytes, is involved in the onset of insulin resistance. As stated above, hypoxia also inhibits adipocyte proliferation. Apparently, cessation of adipocyte differentiation in WAT, together with a increased stress levels due to hypoxic conditions contributes to inflammatory conditions. During the last decade it became clear that adipokines released from the adipose tissue under stress conditions play an important role in the pathogenesis of obesity-related complications (28,38,39).

Currently, many different adipokines are known (27,40-43), albeit with a considerable discrepancy between rodent and human adipokine expression. For instance, adipsin showed decreased expression in obese mice and was originally thought to protect against obesity. In contrast, subsequent human studies failed to confirm this hypothesis and showed increased adipsin levels in obese persons (44). In addition, tumor necrosis factor-alpha (TNF-a) is expressed in mouse adipose tissue and is released into the circulation, while in human it acts locally in the adipose tissue and is not released into the circulation (45). Even more contrasting is resistin that is produced in mouse adipose cells but not in human adipocytes (46). Together, these results show that, despite the value of rodent studies, a one-to-one extrapolation of data from animal models to human adipokine action is inexpedient. It is therefore necessary to focus on human adipokines in order to understand the link between obesity and metabolic complications in man, and particularly the role of adipokines in macrophage recruitment and activation in human WAT.

In obese insulin-resistant subjects, macrophage infiltration into adipose tissue is related to vascular endothelial dysfunction, probably caused by the adipose-tissue inflammatory response (17). To become pro-inflammatory in WAT, macrophages undergo a sequence of events that starts with infiltration as monocytes from the blood into the fat tissue (10,47). Once inside the tissue, monocytes are activated and differentiate into pro-inflammatory macrophages. This process is fuelled by increased expression of pro-inflammatory adipokines, like TNF-a, and an increased release of free fatty acids that target the TLR4 receptor on macrophages (48).

It has been shown that conditioned medium derived from human adipocyte cultures stimulates monocyte diapedesis through endothelial cell layers (49). The same was observed when this medium was de-lipidated to remove adipocyte-released glycerol and fatty acids. In contrast, monocyte diapedesis was blocked when the medium was heated to denature proteins. These results clearly show that human adipocyte-derived adipokines play an important role in monocyte infiltration. Monocyte infiltration involves endothelial cell surface adhesion molecules such as ICAM-1, VCAM-1, PECAM-1, P-selectin and E-selectin (50,51). By the action of adhesion molecules monocytes first attach to the endothelial cell surface and are subsequently arrested by chemokine-activated integrins. Firm adhesion is followed by diapedesis of the monocytes through the endothelial cell-cell junctions (52). To prevent uncontrolled inflammatory reactions, the monocyte entry is strictly controlled by expression of the specific adhesion molecules on the endothelial cell surface (51). Expression of ICAM-1 and PECAM-1 on adipose tissue endothelial cells was increased by secretion medium from human adipocytes, which provided an explanation for the increased monocyte diapedesis (49).

In mice, the monocyte chemoattractive protein 1 (MCP-1) was identified as an adipocyte-derived factor involved in macrophage recruitment (53). However, macrophage recruitment was not impaired in Mcp-1 -/- knock out mice (54,55), suggesting that MCP-1 might not be a critical factor in macrophage recruitment in mice. With respect to humans, increased circulating MCP-1 levels are observed in obese subjects, which could be reversed upon weight loss (56). However, it is not clear whether human adipocyte-derived MCP-1 alters the expression of endothelial adhesion molecules and as such stimulates macrophage recruitment. On the other hand, increased concentrations of recombinant human leptin induced expression of adhesion molecules on endothelial cells and resulted in increased transmigration of monocytes through an endothelial cell layer (49). Together, there is clear evidence that adipokines are involved in macrophage recruitment. However, much is still to be discovered about the key human adipokines that are responsible for induction of macrophage recruitment. We are currently developing a novel method to identify adipokine-receptor interactions. This may be a helpful approach to study intercellular communication and may shed light on human adipokines responsible for macrophage diapedesis.

Intervention of adipose tissue macrophage infiltration

Although thiazolidinediones (TZDs) received negative attention due to the withdrawal of troglitazone and controversial publications regarding rosiglitazone (57), they provided important in-

formation on the role of PPARg as a key regulator for insulin sensitivity and anti-inflammatory mechanisms (58). TZDs antagonize pro-inflammatory conditions by decreasing the expression of T-cell-derived cytokines, endothelial adhesion molecules and endothelial chemokines (59-61). This suggests that TZDs might impair monocyte recruitment in adipose tissue by a modulation of endothelial cells. However, as PPARg agonists, TZDs strongly activate pre-adipocyte hypertrophy (62), which is considered a risk factor for metabolic complications. As such, the effect mediated by TZDs seems paradoxal. Our group provided an explanation for this discrepancy by showing that rosiglitazone reduces adipokine expression and induces a fat-catabolizing metabolism in mature 3T3-L1 adipocytes (63, 64). Whether this also occurs in cultured human adipocytes is unclear. As such, it remains to be discovered whether the action of TZDs on human adipocytes plays a role in the immune-modulating effects of these drugs on endothelial cells.

Adipocyte death

Macrophages particularly surround large adipocytes (2) and the frequency of adipocyte death is positively correlated with adipocyte size (25,65). Furthermore, over 90% of macrophages in mouse and human WAT are localized to dead adipocytes (25). This suggests that adipocyte death is strongly associated with macrophage recruitment.

Adipocyte death in WAT is a physiological process since about 10% of fat cells in adults are renewed annually. However, the total number of adipocytes remains constant in lean and obese, which indicate a dynamic, but tightly regulated, fat cell turnover in man (66). Obese persons have more adipocytes and thus the absolute number of dead adipocytes is expected to be higher (67). This may lead to a sustained macrophage recruitment and development of a chronic inflammatory state.

In mice, the frequency of dead adipocytes in epididymal fat increased in a time-dependent manner during diet-induced obesity for 16 weeks (10). This was coincided with increased fat depot weight, an increased number of infiltrated macrophages, induced expression of TNF-a, MCP-1, IL-10 and induction of insulin resistance. However, after 20 weeks the number of adipocytes was restored and expression of macrophage markers was declined, although the number of dead adipocytes remained constant in subcutaneous WAT. These results suggest that macrophages at first function in adipose tissue remodeling. However, under persistent inflammatory conditions macrophages remain activated and fuse to form multinucleate giant cells (68, 69), a hallmark for adipose tissue macrophages. One can imagine that a deregulation of adipokine expression during adipocyte hypertrophy and hypoxia provides a continuous source of pro-inflammatory cytokines that attract and activate macrophages. A potential additional mechanism is presented here by showing that mature adipocytes subjected to hypoxia expose phosphatidylserine (PS), a molecule that serves as a recognition site for phagocytic macrophages (70).

Membrane lipid asymmetry and phosphatidylserine exposure

The cellular plasma membrane is built up as a lipid bilayer. The majority of this lipid bilayer consists of four phospholipids: phosphatidylserine (PS), phosphatidyl-ethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (Sph) (71). In quiescent cells these are asymmetrically distributed, with PC and SM predominantly present at the outer leaflet and PE and PS mainly present at inner leaflet. The asymmetric distribution of the phospholipids is maintained by the action of three types of lipid transporters: flippases, floppases and scramblase(s) (71, 72), see also Fig. 1. A flippase can 'flip in' PS and PE from the outer to the inner leaflet, against their concentration gradients. For this reason this transporter is also referred to as an aminophospholipid translocase. The half time of PS exchange is 5-10 minutes, for PE this goes at a slower rate. Lipid translocation is an energy consuming process as one molecule ATP is hydrolyzed for every lipid transported. This phospholipid translocation is also present in intracellular membranes, for example mitochondria and the ER. Floppases 'flop out' PC, PS and PE to the outside of the membrane. For this process less ATP is required and it works 10 times slower than the flippase-mediated translocation. The floppase appears to be identical to the ABC transport protein ABCC1 (71).

Flippases and floppases are both constantly in an active state to maintain the bilayer asymmetry. Both make sure that all phospholipids are slowly transported to the outer leaflet, while PS and PE are quickly transported back to the cytosolic side. The third type of lipid transporter is a so-called scramblase, which scramble the membrane asymmetry within minutes, by randomly switching phospholipids from either side of the membrane bilayer to the other side. For long times protein candidates for this type of transport activity remained elusive, but recently TMEM16F was identified as a protein involved in scrambling mouse B-cell membranes (73). Furthermore, a truncated form of this protein was found patients with Scott syndrome, a rare congenital bleeding disorder caused by an impaired platelet procoagulant activity (74). By the scramblase activity, PS is exposed rapidly to the outer surface of the cell. While the other transporters are ATP dependent, the scramblase mainly relies on a high concentration of Ca^{2+} in the cytoplasm, characteristic for apoptotic cells.

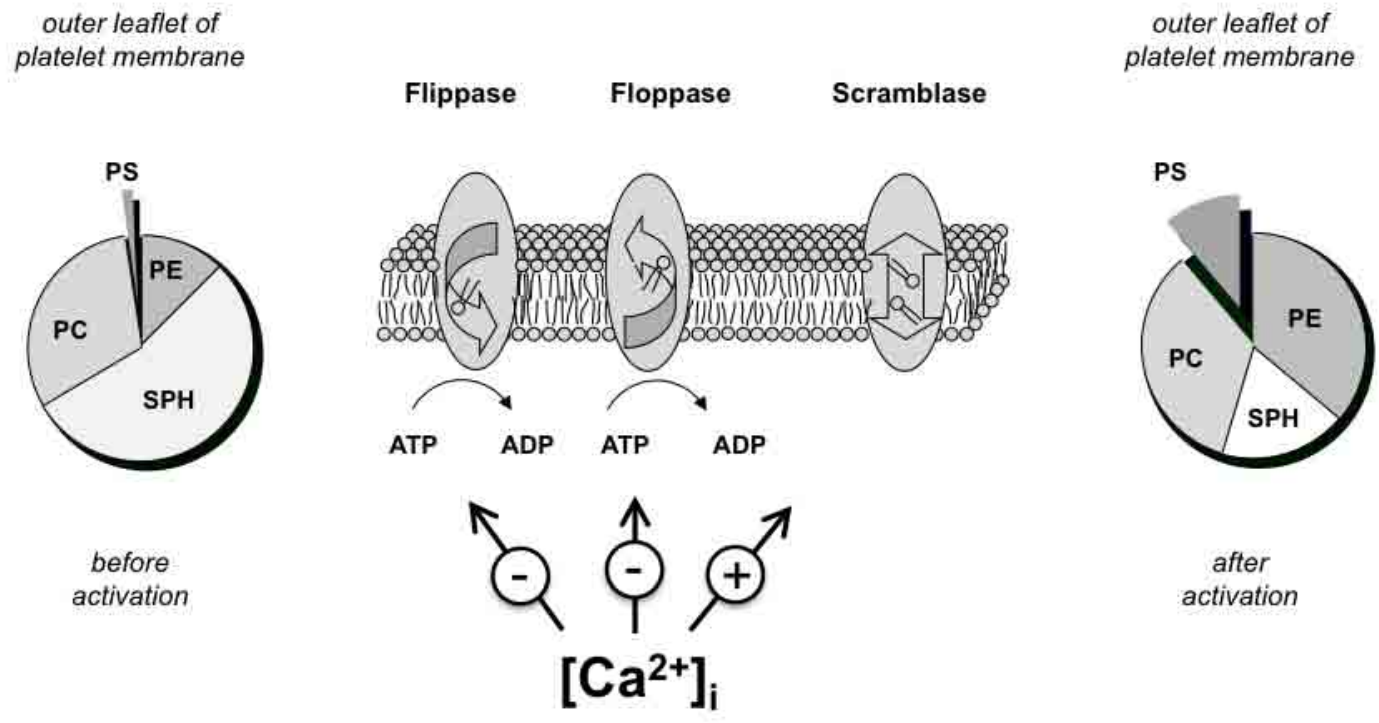

Figure 1. Example of transporter-controlled exchange of phospholipids between leaflets of the platelet plasma membrane. Unidirectional transport of phosphatidylserine (PS) and phosphatidylethanolamine (PE) by flippases is directed inward. Floppases are involved in the outward movement of phosphatidylcholine (PC) and sphingomyelin (SPH). Bidirectional (a-specific) phospholipid transport is catalyzed by a scramblase upon activation with Ca^{2+}. As such, it promotes a collapse of membrane phosphoplipid asymmetry resulting in PS exposure at the outer leaflet. (According to ref. 71)

PS-exposure in physiological conditions

Several physiological processes depend on PS exposure. For instance, platelet activation and coagulation near injuries is PS-mediated. With respect to scavenging of non-functional cells, PS exposure initiates the binding of macrophages, followed by phagocytosis (75). Changes in intracellular ATP and Ca^{2+} are correlated with a collapse of the mitochondrial membrane potential (76). Apoptotic events, which make the mitochondrial membrane permeable, result in the leakage of electrolytes, by which this collapse will be induced. At the same time the permeable membrane results in the leakage of mitochondrial contents, such as ATP and Ca^{2+}, into the cytoplasm, that leads to exposure of PS to the outer leaflet of the membrane. Furthermore, ER-stress leads to PS exposure (77) and ER-stress markers are observed in hypoxic adipocytes (24). As mentioned before, macrophages in WAT surround necrotic adipocytes in crown-like structures, scavenging residual adipocyte material and ultimately form multinucleate giant cells (25). Since macrophages particularly surround dead adipocytes in hypoxic areas it is tempting to speculate that adipocytes subjected to hypoxia expose PS as part of a stress response. As such we hypothesized that PS-exposition is a recognition signal for macrophages to interact with adipocytes that are under hypoxic stress.

PS-exposure in adipocytes

PS exposition in adipocytes has not been demonstrated before. As such, the ability of human adipocytes to expose PS was investigated. For this, human SGBS adipocytes were used and these were incubated with ionomycin, a calcium ionophore that induces a collapse of membrane phospholipid asymmetry, which results in PS-exposure. PS was detected by specific binding of fluorescently labelled Annexin5 at the extracellular site of the plasma membrane. Pre-incubating the cells with EDTA diminished binding of Annexin5, which confirmed that inomycin-induced PS-exposure is Ca^{2+}-dependent (Fig. 2). Subsequently, SGBS cells were exposed to hypoxic conditions during the course of differentiation. PS-exposure was only measured at day 14 of differentiation. No PS-exposure was observed in the control adipocytes (Fig. 3). These results show that adipocytes are able to expose PS. Furthermore, they indicate that only mature adipocytes expose PS under hypoxic conditions.

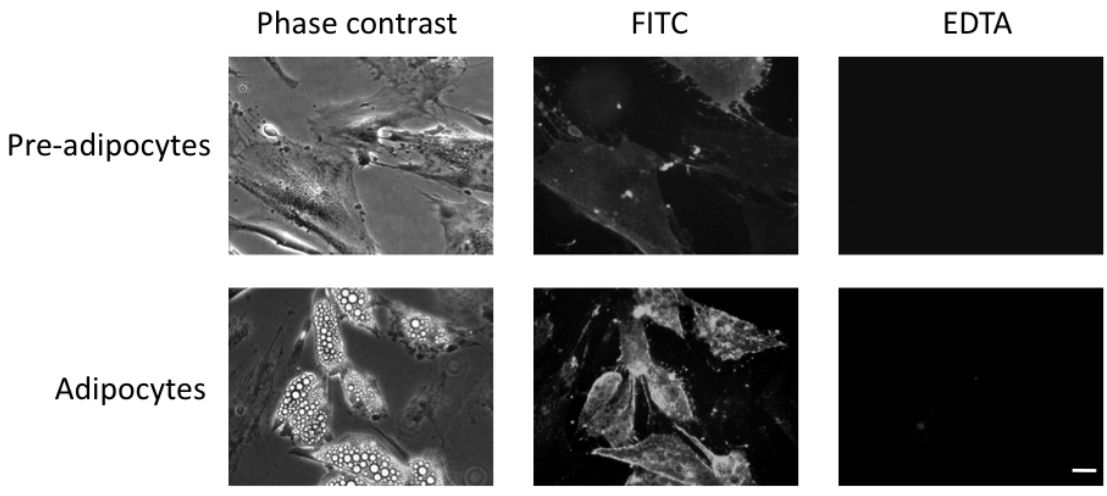

Figure 2. PS exposure in human SGBS (pre)adipocytes. Ionomycin incubation (45 min., 5 µM) resulted in annexinA5-FITC binding to the cells. Binding of annexinA5 was diminished in the presence of EDTA (0.1 mM), showing that this is Ca^{2+}- dependent. Bar indicates 0.25 mm.

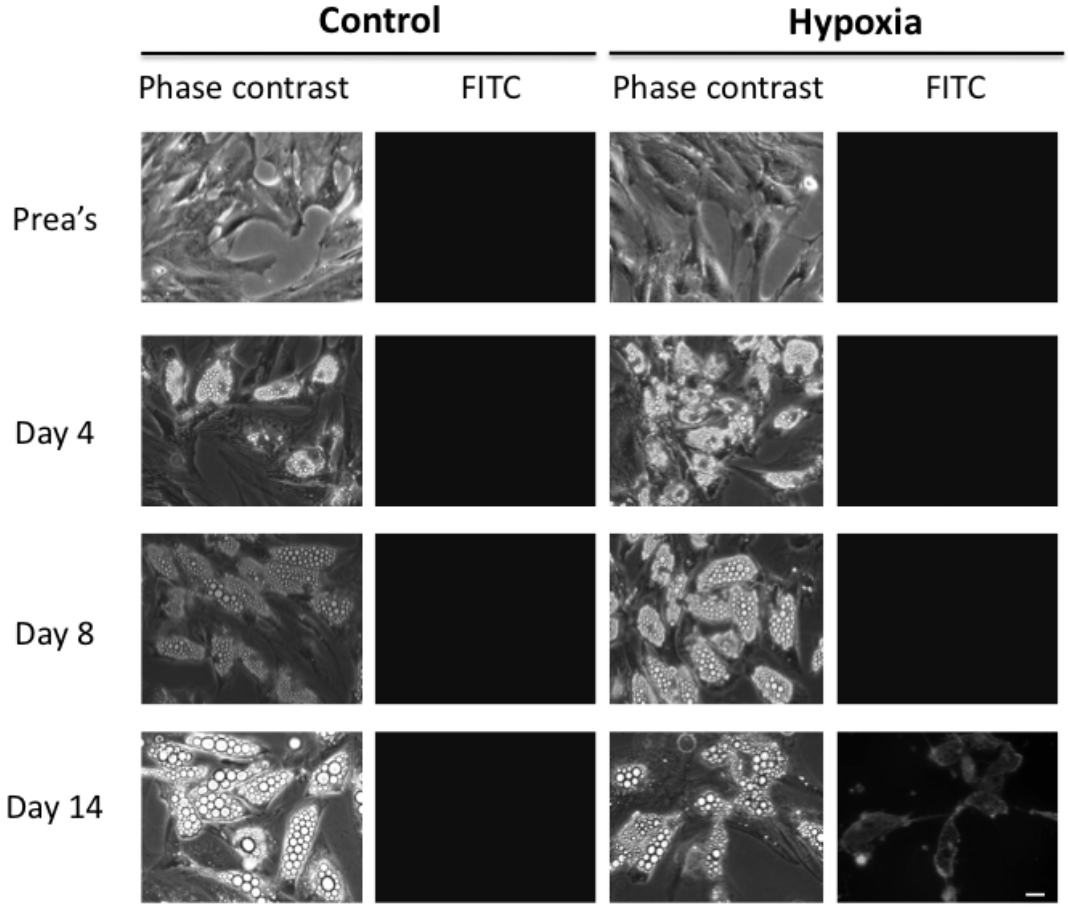

Figure 3. Hypoxia induces PS exposure in mature SGBS adipocytes. SGBS pre-adipocytes (prea's) and adipocytes (4-14 days of differentiation) were subjected to hypoxic conditions (16 hrs., 100 mM $CoCl_2$). After 14 days of differentiation a mixed population of cells was observed with FITC-negative and FITC-positive (~10-20% of the cell population). All FITC-positive cells were also positive for propidium iodide (PI). This demonstrates a loss of membrane integrity. Bar indicates 0.25 mm.

Interaction partners for PS-mediated macrophage docking

There seems no single PS-receptor (78). T-cell immunoglobulin- and mucin-domain-containing molecule 4 (TIM-4) is involved in macrophage binding since antibodies against this protein inhibited apoptotic cell removal (79). In addition, blocking the TIM-4-TIM-1 pathway resulted in decreased macrophage infiltration in murine injured liver tissue (80). Stabilin-2 is identified as another receptor molecule for PS. Blocking of stabilin-1 resulted in impaired PS-mediated clearance of defective erythrocytes by macrophages (81). In recent years it has become clear that docking to PS involves additional molecules that acts as bridging partners between macrophages and PS-exposing cells (78). Growth arrest-specific 6 (GAS6) is a protein that has a high affinity for PS (82), but not for other phospholipids (83). GAS6 is also the ligand for the MerTK receptor (84), a tyrosine kinase receptor expressed on active macrophages. In this way, GAS6 forms a molecular bridge between PS-exposing cells and macrophages. In $GAS6^{-/-}$ mice macrophage diapedesis through endothelial cells was impaired (82). It is known that GAS6 is expressed in 3T3-L1 adipocytes and is secreted via microvesicles (85). The presence of GAS6 in human adipocytes is currently unknown.

Another bridging molecule for PS-macrophage interaction is milk fat globule-EGF factor 8 (MFG-E8) (86). This protein has a PS-binding domain and is secreted by activated macrophages [17,18]. It mediates the adhesion of monocytes expressing the $\alpha_v\beta_3$ integrin to exposed PS via its RGD domain (87). Hanayama *et al* (86) transformed NIH3T3 to express the $\alpha_v\beta_3$ integrin and showed a significantly elevated binding of these cells to PS-coated platelets in the presence of MFG-E8. With a mutant form of MFG-E8 adhesion was not observed, while higher concentrations of MFG-E8 showed increased binding of $\alpha_v\beta_3$ expressing NIH3T3 cells. Apparently, the sole MFG-E8 bridge is enough to induce macrophage-PS interaction. MFG-E8 expression was elevated in hypertrophic adipose tissue of diet-induced obese C57BL/6 mice (88). Together, these results show that PS-mediated macrophage docking is a regulated process involving several different proteins. Some of these are expressed by adipocytes.

Resume: a model for macrophage recruitment, activation and interaction in WAT

To summarize, macrophage recruitment and activation in WAT seems an adipocyte-driven process that involves adipocyte hyperplasia, secretion of pro-inflammatory adipokines and, as shown here, PS-exposure. In the course of obesity the WAT expands by adipocyte hypertrophy and hyperplasia, both events result in a considerable change in adipokine profiles. Adipocyte hypertrophy is associated with hypoxia, most likely to due blocking of blood and oxygen supply. Hypertrophic, but particularly hypoxic adipocytes, secrete pro-inflammatory adipokines, which stimulate macrophage diapedesis. Furthermore, pro-inflammatory adipokines may stimulate macrophages to become activated. Stress on mature adipocytes, either by hypoxia or lipid overload, induces adipocyte death, which is proceeded by PS-exposure. PS behaves as a so-called 'eat-me' signal for macrophages. With the aid of additional bridging molecules macrophages bind to adipocytes to start the phagocytic process, which is fuelled by the release of pro-inflammatory cytokines. Prolonged recruitment and activation of macrophages may thus lead to a chronic inflammatory state that ultimately results in metabolic complications.

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