RESEARCH ARTICLE

Human Adipose Tissue: Purinergic Receptor Involvement

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ABSTRACT

Adipose tissue is derived from the pluripotent mesenchymal cells and further on from cells of the reticular connective tissue, which can produce lobes of fatty tissues like grapes. The reticulum cells store fat droplets that will finally conjoin to one large drop. In the process, the cells become rounder. Extrinsic signaling mechanisms control the cell fate and determination of progenitor cells. Extrinsic signals such as neurotransmitters and neurotrophins have a significant impact on the production and fate or progenitor cells. In addition to intrinsic regulators, extrinsic signaling systems such as neurotransmitters, play a fundamental role in the control of cell proliferation. Purinergic receptors are actively seen at an early stage of development in the germal layer at the embryonic stage.

Aim: This project aims to investigate the presence of purinergic signaling on human adipose tissue. Examine the essential histology of the human adipose tissue through routine histology and scanning electron microscopy.

Materials and Methods: Human adipose tissues were obtained from routine autopsy at AL-Hilla mortuary. Hematoxyline and eosin staining were processed on microtome sectioned slides. Tissues were also examined using scanning electron microscopy SEM. Immunohistochemistry of purinergic receptors (P2Y1, P2Y2, P2Y4) was examined in human adipose tissue.

Results: Histological examination/n of adipose tissue shows mature adipocytes with the lateral location of the nucleus. Purinergic receptors P2Y1, P2Y2, and P2y4 are seen on membranes of adipocytes. PCR product of digested adipose tissues also shows clear bands for all three purinergic receptors in human adipose tissue.

Conclusion: This study shows that mature adipocytes are an excellent source of pluripotent stem cells that are capable of proliferating and differentiating into other cell lineages through purinergic receptor activation using various purine pharmacology. Purinergic receptors are a good indicator of the presence of mesenchymal stem cells.

Keywords: Purinergic receptors, P2Y1, P2Y2, P2Y4

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INTRODUCTION

Adipose tissue is derived from the pluripotent mesenchymal cells, and with time it arises from cells of the reticular connective tissue, which can give rise to grape-like fatty tissue lobes. The reticulum cells are fat droplets storage units that will finally conjoin to form a large drop. During this process, the cells become even rounder. This results in a remarkably large, about 100µm wide, vacuole cells.[1] Their nuclei and cytoplasm are squeezed and displaced to the cell periphery to form what's known as (*signet-ring form*). Usual histological routine preparations (paraffin sections), alcohol and xylene result in the fat being dissolved and removed it from the tissue.[2] This creates causes spaces without stain, which are usually defined as fat vacuoles. Adipose tissue is highly vascularized and containing nerves.

Adult mesenchymal stem cells (MSCs) were mainly isolated from bone marrow through aspiration techniques, but, recently, adult adipose tissue-derived stromal cells (ADSCs) have become the center of attention in reconstructing damaged cartilaginous tissue, this is due to their availability and the ease in harvesting a large number compared to other stem cells sources, since there is no other human tissue expendable as adipose tissue, making it easy to isolate substantial numbers of ASCs for possible human therapies.[1,2]

Regenerative medicine is a fast-growing field and promises to repair damaged tissues and organs and restore function by utilizing the body's regenerative capacity. Due to the availability of MSCs they are becoming an interesting cell source for regenerative medicine and are most commonly isolated bone marrow.[3-6] However, due to the procedures involved in obtaining bone marrow MSCs, they are becoming suboptimal for clinical use due to the invasive aspiration procedure as well as the decline in both their proliferation and differentiation potential with increasing senescence.[7]

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In search of reliable stem cell sources, MSCs derived from adipose tissue introduced a multipotent, undifferentiated, self-renewing progenitor cell population that is morphologically and phenotypically similar to the MSCs provided new hope for regenerative medicine research.

Subcutaneous adipose tissue consists of a heterogeneous stromal vascular fraction (SVF) and predominantly of mature adipocytes. The SVF includes fibroblasts, endothelial cells, pre-adipocytes, vascular smooth muscle cells, monocytes, lymphocytes.[1] The most commonly used method to isolate ADSCs from fat tissue utilizes a collagenase digestion procedure, which is then followed by centrifugal density gradient separation.[7] In vitro, ADSCs display a spindle-shaped morphology lacking the intracellular lipid droplets, as seen in adipocytes histology. Isolated ADSCs are expanded as monolayer cultures on standard tissue dishes with a basal medium containing fetal bovine serum.[1,5-7] However, clinical translation the in vitro expansion of ADSCs has to be compliant with the good manufacturing practice (GMP) guidelines.

There has been significant interest in determining the molecular cues that regulate the proliferation of adult stem cells. Pearson and colleagues[18] examined a number of genes associated with retinal development in lower vertebrates and how they respond to different molecular cues.[19,20]

Extrinsic signalling mechanisms control the cell fate and determination of progenitor cells. Extrinsic signals such as neurotransmitters and neurotrophins have a significant impact on the production and fate or progenitor cells.[18] In addition to intrinsic regulators, extrinsic signaling systems such as neurotransmitters, play a fundamental role in the control of cell proliferation. Purinergic receptors are actively seen at an early stage of development in the germal layer at embryonic stage. Purinergic signaling mediated by nucleotides such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) has also been shown to play a significant role in embryogenesis.[13] Purinergic nucleotides target a group of receptors known as purinergic receptors, which fall into two classes P2Y and P2X receptors; the P2Y receptors are membrane-bound G protein-coupled receptors. When activated, the P2Y receptors produce inositol 1,4,5-trisphosphate (IP_3) and induce calcium movement.[18]

Purinergic signaling has been shown to play a significant role in the regulation of proliferation in subventrical zone of adult mice. The role of P2Y1 and P2Y2 receptors in these neurospheres have been examined through the application of purinergic agonists. The presence of E-NTPDase2 converts ATP to ADP, which stimulates the P2Y1 receptors. Exposure to P2Y1 agonists caused an increase in intracellular calcium, and a significant increase in subventricular cell proliferation increase.[19]

Jia and colleagues[11] reported that activation of purinergic receptors resulted in increased proliferation and subsequent neural differentiation of olfactory epithelium cells. Pearson and colleagues[18] examined the role of purinergic signaling and their role in regulating the cell cycle in the developing chick ventricular zone. The group examined calcium signaling in the ventricular zone of chick retina as a result of purinergic receptor activation. Increases in $[Ca^{2+}]$ as a result of purinergic receptor activation, had a dramatic influence on the rate of proliferation of mitotic cells in the ventricular zone. Thus intracellular calcium has a significant role on the cell cycle of progenitor cells in the chick ventricular zone, and that neurotransmitters can act to modulate cell cycle progression.[19,20]

How intracellular calcium influences the cell cycle is unclear. However, changes in intracellular calcium concentration in retinal progenitor cells has been shown to affect the interkinetic movement of cycling retinal progenitor cells. The division of progenitor cells during early development is essential to expand the progenitor pool. The migration of progenitor cells between the proliferative ventricular zone and the inner retina during retinal development is known as interkinetic nuclear movement.[18] Division of cells occurs at the ventricular surface needing various factors to increase the nuclear movement. Pearson et al. demonstrated that changes in intracellular calcium are important for the maintenance of the nuclear movement of cells. The application of gap junctions blockers resulted in a slowing migration of the progenitor cells. Since retinal progenitor cells express purinergic receptors (P2Y) and that their activation by ATP results in significant changes in $[Ca^{2+}]$, the group have suggested that interkinetic nuclear movement of progenitor cells might also involve purinergic regulation.[17-19] ATP is released by Retinal pigmented epithelium cells via gap junctions hemichannels, stimulating mitosis in the progenitors. The progenitors are also connected via gap junctions and different mechanisms.

Pearson and colleagues[18-20] further investigated the impact of ATP on neural retinal progenitor proliferation. They found that ATP released from gap junctions hemichannels in the Retinal pigmented epithelium cells and resulted in the elevation of $[Ca^{2+}]$ ions in the underlying retinal progenitor cells. The release of ATP is localized to the surface of the retinal pigmented epithelium cells that face the neural retina indicating a strong impact on the development of the neural retina. The group showed that the release of ATP activates the purinergic receptors present on retinal progenitor cells, evoking an increase in $[Ca^{2+}]$ ions and subsequent stimulation of mitosis. The application of purinergic antagonists has been shown to decrease the rate of mitosis in progenitor cells in the neural retina. The group showed that there is a significant role played by ATP in the regulation of retinal progenitor cell proliferation. Furthermore, the group showed that the release mechanism of ATP from the retinal pigmented cells is an essential process during neural retina development.[17,20]

The role of purinergic signaling extends to the regulation of other regional neural development. The role of ATP signaling and neural development in various tissues. ATP signaling has been shown to have a significant impact on developmental patterns in various tissues. As shown above,[16] the retinal development is highly mediated by waves of calcium ions triggered by ATP receptor activity, evoking a wave of

increase calcium ions, which impacts the rate of progenitor cell division. In the developing chochlea, a small structure known as the kollicker's organ is present during the early stages of development.6 Recent studies showed that such structure poses electrical potential mediated by the presence of P2Y and P2X receptors and through their release of ATP, which evokes calcium ion waves as a result of their activation. ATP release induces hair cell depolarization resulting in a spontaneous release of glutamate in the kollicker's organ.

Similarly, purinergic signaling is important in the regulation of vascular cell proliferation. Adenosine, an enzymatic breakdown of ATP, has been shown to regulate smooth muscle cell proliferation. Cultured vascular smooth muscle cells have been shown to proliferate and undergo DNA synthesis through the application of ATP and ADP and their activation of P2Y receptors.[13]

Purinergic signaling has been shown to play a significant role in the development of a number of tissues. The presence of the purinergic receptor in numerous embryonic mammalian tissues indicates an important role in cell proliferation and normal development. It has also been reported to regulate and maintain the proliferation of adult stem cell populations, including those found in the olfactory bulb.[9,11] A recent study by khalfa and colleagues[24] showed the involvement of purinergic receptors in the human epidermis at different stages of development. Their finding showed that epidermal development is highly driven by the presence of purinergic receptor.[25]

MATERIALS AND METHODS

Human adipose tissue

Adult liposuction samples were obtained during routine autopsy examination, and a legal consent was obtained from both Baghdad and Hilla mortary/Iraq. A total of 3 male adult tissues were used for this study. Any adult suffering from abdominal disease or defects are not included in this study.

Histological processing

All samples were fixed in 10% formaldehyde for 24 hours and then processed for routine paraffin embedding.

Tissue embedding

All tissues were embedded in paraffin at 6°C for routine Hematoxylin, Eosin staining, or in OCT (optimum cutting temperature) for immunohistochemistry. The issues were sectioned at 4 µm and collected on charged slides for immunohistochemistry.

Immunohistochemistry of P2Y1, P2Y2 and P2Y4 receptors on human adipose tissue

Fresh adult adipose tissues were washed in PBS before embedding in OCT (optimum cutting temperature) and placed on metal chucks. Tissues were sectioned at 4 µm in -24°C, then collected on charged microscopic slides. Slides were allowed to air dry for 30 minutes before immunohistochemistry. Immunohistochemistry for purinergic receptor P2Y1, P2Y2 and P2Y4 was carried out using manufacturer protocol.

Polymerase chain reaction (PCR)

The PCR reaction was performed using primers for P2Y1, P2Y2, and P2Y4 receptors (purchased from IDT) table below. DNA extraction was achieved using one-step DNA extraction kit purchased from (Bioneer/Korea). Tissue digestion and DNA extraction was achieved following the manufacturer protocol.

1µ of left and right Primers for each receptor were used balanced with 9.5µL of water and 12.5µ of mater mix (Bioneer, Korea). The above quantities were mixed in PCR tubes, and 1µ of CDNA was added. Denaturation was performed at 95 C for 5 minutes, followed by 40 cycles at 95c for 30 s, at 54 C for 40 s and at 72C for 1 minutes, with a final elongation step at 72C for 7 minutes. Digestion products were analyzed on 3% agarose gel.

RESULTS AND DISCUSSION

The results of the current study show that human adipose tissue is a versatile tissue which easily obtained from various surgical procedures. It can act as a source for stem cells and cellular research.

Human adipose research is a widely growing field since they are easily harvested through a surgical procedure. Conventional histological appearance of adipocytes in clusters suspended in loose connective tissues were seen in this study which agrees with all histological descriptions of human adipose tissues. A clear cytoplasmic rim is seen with nuclear displacement to a peripheral location in the cells (Figure 1). SEM Micrograph of adipose tissue shows clustered cells with loose connective tissues attached to the cells (Figure 3). Clusters of adipocytes appear disorganized, possibly due to the harsh SEM processing materials, which is expected due to the materials used and the immersion of tissues in

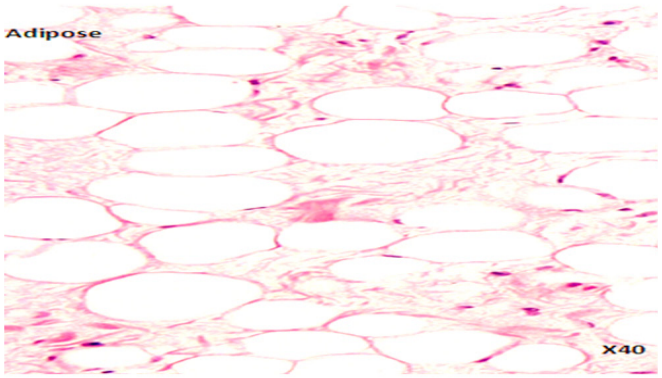

Figure 1: Histological (H&E) staining of human adipose tissue X40. Adipocytes are seen in clusters suspended in loose connective tissues. The clear cytoplasmic rim is seen with nuclear displacement to peripheral location.

Primers	*Left*	*Right*	*Product length (bp)*
P2Y1	Ctgtgtggaccccattcttt	tcgggacagtctccttctga	439
P2Y2	Gagcatcctcaccacctca	gctattccagggttccaggt	634
P2Y4	gaagaagcagcagaacacca	caaggagtctgcactggtca	319

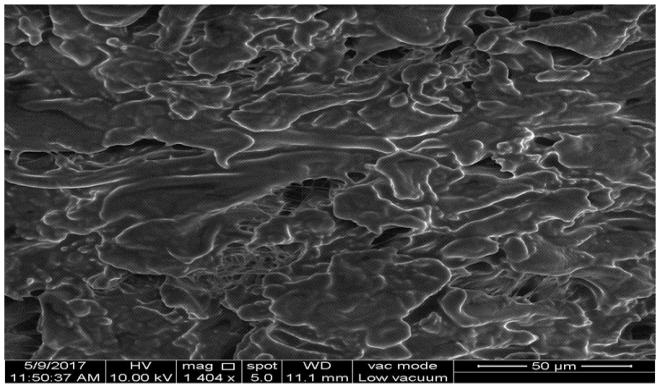

Figure 3: Electron micrograph of an adult adipose tissue X 1404. The image shows highly clustered cells with loose connective tissues attached to the cells. Clusters of adipocytes appear disorganized possible due to the harsh SEM processing materials.

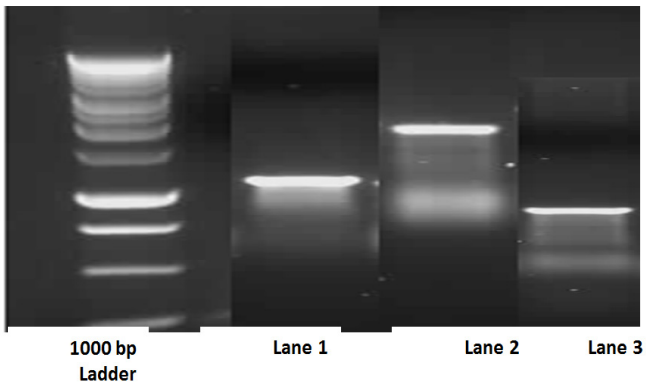

Figure 2: Gel electrophoresis of Purinergic receptors in adipose tissue. The bp ladder is used to analyze the PCR product. Lane 1 shows a band corresponding to P2Y1 receptors at around 400 bp. Lane 2 shows a band corresponding to P2Y2 receptors at 600 bp. Lane 3 shows the PCR product of P2Y4 at 319 bp.

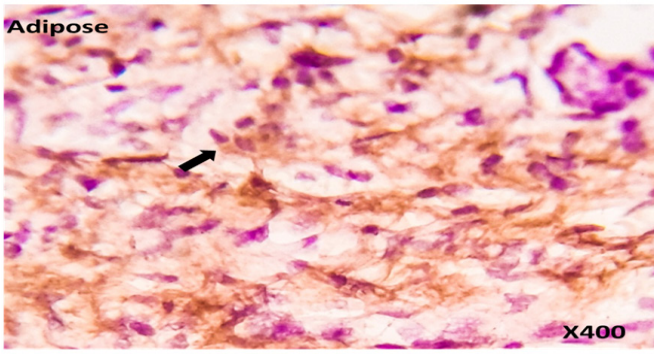

Figure 4: Human adipose tissue shows a positive stain for P2Y1. Positive staining is seen on membranous bound location (Arrow) on adipocytes indication of proliferating cells and possible stem cells.

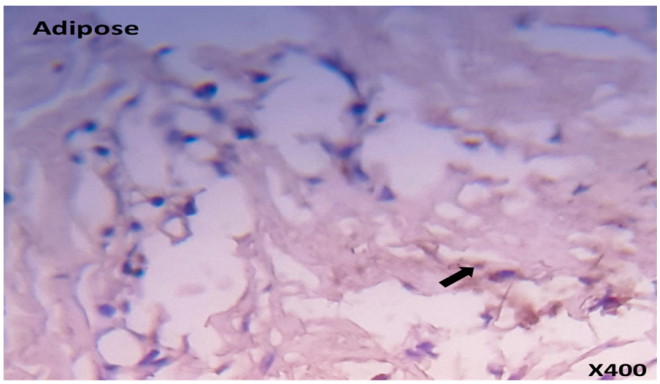

Figure 5: human adipose tissue shows a positive stain for P2Y2. Positive staining is seen on membraneous (Arrow) bound location indicating possible stem cell existence and high proliferating cell activity.

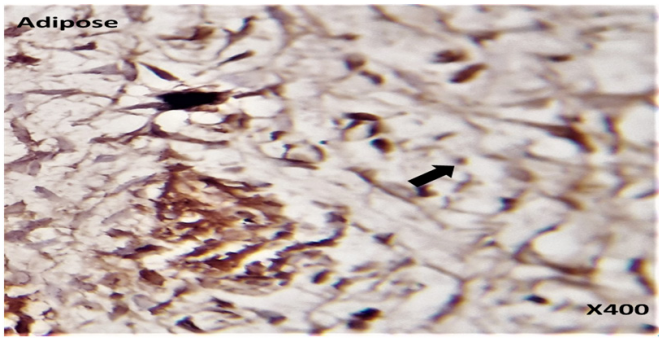

Figure 6: human adipose tissue shows a positive stain for P2Y4. Weak Positive staining is seen on membranous bound location (Arrow), indicating possible stem cell existence and some proliferating cell activity.

ethanol. A polymerase chain reaction of all three purinergic receptors was seen at their corresponding band sizes Lane 1 shows a band corresponding to P2Y1 receptors at around 400 bp. Lane 2 shows a band corresponding to P2Y2 receptors at 600 bp. Lane 3 shows the PCR product of P2Y4 at 319 bp (Figure 2). To further examine the location of purinergic receptors on adipocytes immunohistochemical examination was performed. Strong P2Y1 positive staining was seen on membranes of adipocytes (Figure 4). Positive staining for P2Y2 receptors was seen on membranes of adipocytes (Figure 5) which is expected according to previous work on purinergic receptor expression on various human tissues.[25] Weak P2Y1 positive staining was seen on membranes of adipocytes (Figure 6). Studies by various researchers have established d the presence of purinergic receptors as indicators for the presence of stem cells.[8,11,12,16,18,25] Their activation does lead to many biochemical and cellular responses as shown in various human tissues such as the retina and the olfactory bulb.[9,12,16,25] Genetic and histochemical results of purinergic receptors in this study provide further evidence to the presence of stem cells in human adipose tissue.

CONCLUSION

The outcome of our study provides distinct evidence that human adipose tissue possesses stem cell characteristics by expressing purinergic receptors that drive molecular cascades essential for cell proliferation and differentiation. Human purinergic receptors were expressed on membranous locations of adipocytes. Furthermore, their genetic expression was detected through PCR and their corresponding band size was detected correctly according to previous research.

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