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Adipose Tissue-Derived Stem Cells from Affected and Unaffected Areas in Patients with Multiple Symmetric Lipomatosis show Differential Regulation of mTOR Pathway Genes

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Abstract

Background: Multiple symmetric lipomatosis is a rare disease characterized by the excessive growth of uncapsulated masses of adipose tissue. Although the etiology has yet to be elucidated, a connection to brown adipose tissue has been proposed recently. The mTOR pathway, which is found to be regulated in lipomatous tissue as well as associated with brown adipose tissue, can be inhibited by a compound called rapamycin.

Methods: We isolated adipose tissue derived stem cells from both affected and unaffected tissue and treated these cells with different concentrations of rapamycin.

Results: The differences in both proliferation and differentiation between adipose tissue derived stem cells (ASCs) from lipomatous and normal tissue decreased after mTOR pathway inhibition. In some patients regulation of mTOR genes was opposed in the ASCs from the two different tissues.

Conclusions: Treatment with rapamycin might be a novel therapeutical approach for patients suffering from multiple symmetric lipomatosis.

Key Words

Multiple Symmetric Lipomatosis, rare disease, mTOR pathway, rapamycin
1. Introduction

1.1 Multiple symmetric lipomatosis

The rare disease (estimated incidence 1:25,000) multiple symmetric lipomatosis (MSL; also benign symmetric lipomatosis) is of unknown etiology and characterized by expansive growth of adipose tissue. Patients suffering from MSL can be divided into different phenotypes. Type I (horsecollar lipomata) includes exaggerated fat distribution in the neck, upper back, shoulders and upper arms; type II (pseudoathletic appearance) involves exaggerated fat distribution in the shoulder girdle, deltoid region, upper arms, and the thorax; type III (gynecoid distribution) is related to an excess of lipomatous tissue in the lower body, especially the thighs and medial side of the knees [1]. A fourth type (abdominal type) has been proposed. MSL was first described by Brodie in 1846 and further characterized by Madelung in 1888 and Launois and Bensaude in 1898 [2-4]. MSL is often associated with diabetes mellitus, hyperlipidaemia, hyperuricaemia, hypothyroidism, neural pathologies [5, 6] and the MERRF syndrome (myoclonic epilepsy with rigid red fiber) or other mitochondrial diseases [7, 8]. MSL may cause severe complications due to tracheal, laryngeal or mediastinal compression [9-12]. This could lead to sleep apnea [13]. It has been reported that MSL patients with no signs of coronary artery disease, acute myocardial infarction, or other cardiac abnormalities have suffered from sudden cardiac death [14, 15]. The authors have linked this mortality to the occupation of the mediastinal space by the lipomatous tissue. Although elevated alcohol consumption is common in patients suffering from MSL, many cases are reported where patients did not drink any alcohol at all [16]. Additionally, the neuropathies cannot be explained solely by the elevated alcohol consumption found in most MSL patients [17, 18]. Whereas most cases are sporadic, some familial cases (inherited in an autosomal-dominant fashion) have been reported [19-21]. However, the underlying genetic cause is unknown. An association with brown adipose tissue (BAT) has been proposed recently [16, 22]. This is in accordance with the association of MSL with mitochondrial diseases, because brown fat is a tissue rich in mitochondria [23, 24], and the areas generally affected by MSL mirror the distribution
of BAT [22, 25]. Additionally, adipose tissue derived stem cells (ASCs) from lipomatous tissue were
found to express UCP-1, a marker for brown fat [16, 22, 26].

### 1.2 mTOR signaling pathway

Fat tissue homeostasis is associated with the mTOR (mechanistic target of rapamycin) signaling pathway. The pathway is important for lipid homeostasis [27] and energy homeostasis [28-30]. Additionally, a sustained activated mTOR pathway can be caused by overfeeding and can result in obesity and insulin resistance [31, 32]. Interestingly, insulin resistance is also common in patients suffering from MSL [33-35]. The mTOR gene encodes for a phosphatidylinositol kinase that, amongst others, mediates cellular responses to nutrient deprivation. Activated mTOR pathway results in the phosphorylation of activation of Ribosomal protein S6 kinase beta-1 (RPS6KB1, also p70S6 kinase, p70S6K, p70-S6K) which in turn phosphorylates S6 ribosomal protein, an activation crucial for protein synthesis. Interestingly, it was found that RPS6KB1 is important for early adipocyte differentiation [36, 37]. Using a reverse phase protein assay, we have found that genes that are regulated differentially in adipose tissue derived stem cells (ASCs) from lipomatous tissue compared to ASCs from unaffected fat tissue are overrepresented in the mTOR pathway. Among these genes were IGF, PI3K, and Akt (unpublished data).

### 1.3 Rapamycin

The mTOR pathway can be inhibited by rapamycin (sirolimus). Rapamycin is a compound first found in bacteria (Streptomyces hygroscopicus) from the Easter Island (Rapa Nui) [38]. Rapamycin inhibits the expression of interleukin 2 and therefore the activation of B cells and T cells and is used as an immunosuppressant after organ transplantation. Additionally, rapamycin is used as treatment for lymphangioleiomyomatosis, and stents are coated with rapamycin to suppress the proliferation of smooth muscle cells [39, 40] while the proliferation of endothelial cells is suppressed also [41].

Recently, we have shown that ASCs isolated from affected and unaffected areas can be used as a model system to evaluate differences in the regulation [24]. Additionally, we have seen that many genes regulated differentially in ASCs isolated from affected areas from MSL patients are associated with the
mTOR pathway, which can be inhibited by rapamycin. Here, we show that ASCs from normal and lipomatous tissue react differently to rapamycin treatment and that genes associated with the mTOR-pathway are regulated differentially in these ASCs.

2. Methods

2.1 Cell culture

Cells from fat tissue of affected and unaffected areas from five patients were isolated as previously described [24, 42]. Briefly, surgical biopsies from lipomatous tissue and normal adipose tissue were obtained. All patients were given verbal and written information, and signed informed consent was obtained prior to study start. The study was approved by the Independent Ethic Committee of the University of Regensburg (No.: 08/117). Subcutaneous adipose tissue (10g±0.5g) was harvested under local superficial skin anesthesia with Xylocaine. Tissue sample was used for extraction of adipose tissue derived stem cells (ASCs). The fat tissue was minced into small pieces (1mm³) and subsequently digested with collagenase (Sigma-Aldrich, St. Louis, MO, USA; 5 U/ml fat tissue) for 45 minutes. The solution was filtered through a 100-µm strainer, centrifuged (500 rcf) and the cells from the obtained pellet were seeded into culture flasks with MEM alpha1 medium (Sigma Aldrich) containing 20% heat-inactivated FBS (Pan-Biotech, Aidenbach, Germany), 2 mM glutamine (Thermo Fisher, Waltham, MA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) and were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Upon reaching subconfluency, cells were detached with a 0.25% trypsin/0.02% EDTA solution (Pan-Biotech) and seeded at a density of 5000 cells/cm². Cells in passage 5 were used for all experiments. For adipogenic differentiation, the cell culture medium was additionally supplemented with 1 µM dexamethasone, 0.5 mM IBMX, 100 µM indomethacin, and 10 µg/ml human recombinant insulin (all Sigma-Aldrich). For inhibition of the mTOR signaling pathway the medium was supplemented with rapamycin (Sigma Aldrich, 0 ng/ml, 1 ng/ml, or 10 ng/ml, respectively). After a time period indicated in the respective result section, the cells were either used for vitality assays, stained for detection of adipogenic differentiation, or harvested for RNA isolation.
2.2 Cell viability assay

Cell viability was assessed using resazurin (Sigma-Aldrich) which is metabolized to the fluorescent resorufin by vital cells. ASCs were seeded into 96-well plates and allowed to adhere for 24h. Subsequently, the proliferation medium was discarded and replaced by medium supplemented with different rapamycin concentrations. After the time indicated in the respective experiments, the medium was replaced with a 0.07 mM resazurin in proliferation medium-solution. After 2 hours fluorescence intensity (excitation 530 nm, emission 590 nm) was measured using the VarioScan plate reader (Thermo Fisher). Results are shown as mean of all five patients ± SD.

2.3 Oil red O staining

Staining with Oil red O was used to assess adipogenic differentiation. Cells were cultured until subconfluency was reached. Subsequently, the medium was changed to adipogenic differentiation medium. After ten days, cells were fixed with a 10% formalin (Sigma Aldrich) solution for 10 minutes. After washing with PBS, a 5 mM oil red O in 60% isopropanol solution was added and the cells were stained for 20 minutes. For quantification, the dye was discarded and the cells were washed with PBS four times and with 60% isopropanol one time. Subsequently, oil red O was eluted from the cellular lipid droplets with 100% isopropanol. Optical density at 518 nm wavelength was measured using the VarioScan plate reader. Results are shown as mean of all five patients ± SD.

2.4 Real-Time RT-PCR

Cells were seeded into 6-well plates and upon subconfluency treated with the different rapamycin concentrations for seven days. For RNA isolation, the RNeasy mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s instructions. RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher) and reverse transcription of 1 µg total RNA was done using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was done using the DyNaMo Color Flash SYBR Green Master Mix (Thermo Fisher) and the Eco Thermal cycler (Illumina, San Diego, CA, USA). Primer sequences are as follows: GAPDH_forward: 5’-GAAAGATGGTGATGGGATTTC-3’, GAPDH_reverse: 5’-GAAGGTGAAGGTCGGAGTC-3’; mTOR_forward: 5’-CGAAGCCGCGCGAACC-3’,
mTOR_reverse: 5’-ATTCCGCTTTAGGCCAC-3’; EIF4EBP1_forward: 5’-GGAGTGTCGGAACTCACCTG-3’; EIF4EBP1_reverse: 5’-ACTGTGACTCTTCACCGCC-3’; RPS6K1_forward: 5’-TGTCGACAGCCCAGATGACT-3’, RPS6K1_reverse: 5’-ATTTGACTGGGCTGACAGGT-3’. Experiments were done in triplicates. The ΔΔCt-method was used to calculate relative gene expression with GAPDH as housekeeping gene. Results are shown as mean ± SD for every patient. Relative gene expression is normalized to ASCs.

2.5 Statistics

Statistics was done using Student’s T-Test. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

3. Results

3.1 Cell viability assay

ASCs from lipomatous tissue showed higher cell viability when compared to ASCs from normal tissue. Treatment with rapamycin for 48 hours led to a decrease in cell viability in both 1 and 10 ng/ml concentrations in the ASCs from both normal and lipomatous tissue. After treatment with rapamycin in both concentrations the cells from the unaffected areas had around 65% of their original viability remained. In contrast, cell viability of ASCs from affected tissue decreased to around 35% of their former value (figure 1).

![Cell vitality graph](image)

Figure 1: Normalized cell vitality after 48 hours of ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Results are shown as mean of all five patients ± SD. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.
3.2 Oil red O staining

ASCs from normal tissue showed a higher degree of differentiation when compared to ASCs from lipomatous tissue. Adipogenic differentiation was impaired in ASCs from normal tissue when rapamycin was added to the culture medium. After treatment with 1 ng/ml and 10 ng/ml rapamycin, Oil Red O staining decreased to 91% or 88% of the original values, respectively. Adipogenic differentiation of ASCs from lipomatous tissue was nearly unaffected (Figure 2).

Figure 2: Adipogenic differentiation of ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were differentiated for 10 days and stained with Oil Red O subsequently. After washing, the dye bound to triglycerides was eluted with isopropanol and measured colorimetrically. Values are normalized to undifferentiated ASCs from normal tissue. Results are shown as mean of all five patients ± SD. No statistically significant differences were observed.

3.3 Real-Time (RT)-PCR

Gene expression was regulated differentially in ASCs from affected and unaffected areas after rapamycin exposition in some patients. This was most considerable for the direct target of the mTOR signaling pathway, RPS6K1, but also seen in some patients for EIF4EBP1 and mTOR. RPS6K1 was downregulated with increasing rapamycin concentrations in ASCs from normal tissue but upregulated in ASCs from lipomatous tissue (Figures 3b-e). Patient 1, however, did not show this opposed regulation (Figure 3a). EIF4EBP1 was downregulated or not regulated with increasing rapamycin concentrations in ASCs from unaffected tissue but upregulated in ASCs from affected tissue in some
patients (Figures 4a, d). Patients 2, 3, and 5, however, did not show this opposed regulation (Figures 4b, c, e). mTOR was downregulated with increasing rapamycin concentrations in ASCs from normal tissue but upregulated in ASCs from lipomatous tissue (Figures 5b, c). Patients 1, 4, and 5 however, did not show this opposed regulation (Figures 5a, d, e).

Figure 3: Relative gene expression of RPS6K1 in ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized to undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

4. Discussion

Surgical removal of lipomatous tissue via lipectomy or liposuction provides to date the only validated therapeutic approach for patients suffering from MSL [16]. However, relapse after surgery is occurring frequently [43, 44]. Although the term benign symmetrical lipomatosis implies a mere aesthetic/cosmetic problem, the patients not only suffer from severe psychological strain but also from accessory symptoms that can range from decreased quality of life (tracheal, laryngeal or mediastinal compression, sleep apnea) to sudden cardiac death. Because of this, the justification of the term
benign in benign symmetrical lipomatosis has been doubted [14]. Therefore, a pharmaceutical therapy would be beneficial. In the recent past increasing evidence that BAT might be involved in the etiology of MSL has been reported. The mTOR pathway is associated with lipid homeostasis in general and in particular with the regulation of BAT. Additionally, we have found several genes associated with the mTOR pathway to be regulated differentially in ASCs from lipomatous tissue. Therefore, we have conducted a study to evaluate the influence of the mTOR pathway inhibiting compound rapamycin on ASCs isolated from unaffected and affected tissues from patients suffering from MSL. It was shown that ASCs from lipomatous tissue have a higher proliferation rate but an impaired differentiation capacity when compared with normal tissue ASCs. This is in accordance with the conclusion, that MSL is a hyper proliferative stem cells disorder [26]. After treatment with rapamycin, the cells from the two different tissue origins aligned their proliferation and differentiation behavior. Cell viability was much more impaired in ASCs from lipomatous tissue than in those from unaffected areas. As a result, both cell types had a similar viability (Figure 1). Differentiation on the other hand was impaired significantly in normal tissue ASCs but not in ASCs from lipomatous tissue, making both types becoming more alike (Figure 2). Interestingly, this might at least in part be due to the opposite regulation of mTOR pathway related genes in these ASCs. Real Time RT-PCRs have shown that in some patients, the two different cell types showed a contrary regulation of mTOR, EIF4EBP1, and RPS6K1 when treated with rapamycin. In some patients, after rapamycin incubation these genes are downregulated or not regulated in ASCs from normal tissue but upregulated in ASCs from lipomatous tissue (Figures 3-5).
Figure 4: Relative gene expression of EIF4EBP1 in ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized to undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.
Figure 5: Relative gene expression of mTOR in ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized to undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

However, this regulation was not observed in ASCs from all patients. On the other hand, nearly no accessory symptoms and causes that have been proposed for MSL can be applied to all patients. This has already been reported for alcohol abuse, neuropathies, and for mitochondrial genome mutations, which can be seen in many but not in all patients suffering from MSL [45, 46]. Although additional research is needed to address this question, we can present some evidence to support the hypothesis that mTOR is involved in the pathogenesis of MSL. First of all, mTOR plays a crucial role in lipid homeostasis and adipocyte maturing [47], which might be impaired in ASCs from affected tissues. Moreover, mTOR pathway is involved in BAT regulation, from which lipomatous tissue has been proposed to origin [16, 22, 25, 26]. Lbk1, a gene known to control BAT expansion and UCP-1 expression in mice, exerts is function partly via mTOR signaling pathway [48]. Deletion of mTOR induced white adipose tissue to adopt characteristics from BAT [49]. However, negative impacts of rapamycin on UCP-1 expression in BAT have been reported, too [50]. Associated with the mTOR pathway is Sestrin2, a regulator of thermogenesis in BAT [51]. Moreover, in a previous study we have seen that the proteins found regulated differentially in ASCs isolated from affected areas are overrepresented in the mTOR signaling pathway (IGF, PI3K, Akt). Interestingly, an association of MSL with the mTOR pathway related gene PTEN has been proposed earlier [52-54]. Another connection between mTOR pathway and MSL is insulin resistance, which is common in patients and can result from both overfeeding and a sustained active mTOR pathway [31-35]. Interestingly, both caloric restriction and rapamycin have a positive effect on murine lifespan [55, 56]. Whether the longer life expectancy of mice treated with rapamycin is comparable with the life-extending effects of dietary restriction is yet to be elucidated. However, humans cannot be compared with rodents regarding the positive effects on lifespan of caloric restriction [55, 57]. Rapamycin is already used for several indications. The drug is marketed under the
trade names Rapamune® (Pfizer) or Certican® (Novartis), respectively. Rapamycin, in a medical background often called sirolimus, is used as an immunosuppressant to prevent rejection after kidney, liver, or heart transplantation and is also used as a treatment for renal cell carcinoma [58-62]. Additionally, Sirolimus is used for coronary stent coating [39]. Additionally to these indications, orphan designation was granted by the European Commission for sirolimus for the treatment of chronic non-infectious uveitis (EU/3/11/898, Santen Oy, Finland, 30 August 2011), for the prevention of arteriovenous access dysfunction in patients undergoing surgical creation of an arteriovenous access for hemodialysis (EU/3/13/1204, S-Cubed Limited, United Kingdom, 13 November 2013), for the treatment of tuberous sclerosis (EU/3/15/1557, Desitin Arzneimittel GmbH, Germany, 9 October 2015), and for the treatment of beta-thalassaemia intermedia and major (EU/3/15/1585, Rare Partners srl Impresa Sociale, Italy, 14 December 2015). Here, we show a possible novel therapeutic approach for future therapies of patients suffering from MSL.

5. Conclusion

The mTOR pathway might be involved in the pathogenesis of MSL. Rapamycin could be an approach to a novel pharmaceutical therapy. As already reported for alcohol abuse and for mitochondrial genome mutations [45, 46], this is not necessarily true for all patients.

5.1 Competing interests

We declare that there are no competing interests.

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This paper is dedicated to the 70th birthday of Prof. Friedrich Jung.


References


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