Adipose-Derived Stromal Cells from Lipomas: Isolation, Characterisation and Review of the Literature

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Key Words

Adipocytes · Lipomatosis · Proliferation · Stem cells · Tissue engineering

Abstract

Objective: The aim of this study was to characterize adipose-derived stromal cells (ADSCs) from patients diagnosed with multiple symmetric lipomatosis (MSL) in order to obtain potentially new insights into the pathophysiology, pathogenesis and treatment of this disease. **Methods:** Cells from the stromal vascular fraction were analysed by the colony-forming efficiency assay and flow cytometry using standard markers. Moreover, the power of adipogenic plasticity was evaluated. Finally, a literature review was performed from 1982 to 2015 using the US National Institutes of Health's PubMed database. **Results:** Three European-descent patients diagnosed with either MSL type I or II could be identified for analysis. The resulting mean colony-forming efficiency assay was $14.3 \pm 5\%$. Flow-cytometric analysis of the ADSCs revealed high levels of CD34 ($70 \pm 9\%$), CD45 ($37 \pm 13\%$) and CD73 ($55.8 \pm 14\%$), whereas low levels of CD31 ($16.8 \pm 14\%$) and CD105 ($5.8 \pm 0.7\%$) were detected. Furthermore, ADSCs showed a strong adipogenic potential, which is in line with the literature review. The stem cell pool in lipoma shows several alterations in biological activities, such as proliferation,

apoptosis and stemness. **Conclusions:** ADSCs from lipoma may be interesting in the application of regenerative medicine. We discuss possible molecular treatment options to regulate their activities at the source of the MSL.

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Introduction

Lipoma is one of the most common soft-tissue mesenchymal neoplasms in adults and can be located in any part of the body [1]. Multiple symmetric lipomatosis (MSL) is a rare disorder and largely more prevalent in males of Mediterranean descent [2]. The disease can be defined as type I MSL if the fatty tumours maintain the aspect of distinct, well-circumscribed, grossly round masses protruding from the body surface [3]. Type II disease is characterized by extensive, diffuse involvement of lipomatous tissue in the subcutaneous fat layer, giving the patients the appearance of simple obesity [3].

Details of the cellular and molecular phenomena underlying the pathogenesis of this disease remain unclear, although there are reports of proliferative activity and chromosomal aberrations of lipoma adipocytes from the brown adipose tissue [4, 5]. Furthermore, an association between metabolic disorders and alcoholism has been

postulated [6–8]. Interestingly, it has been reported that chemotherapy and/or blood stem cell transplantation may be plausible causes of de novo multiple lipomatosis, possibly by stem cell mobilization following transplantation or chemotherapy [9]. Lastly, there is an association between the myoclonus epilepsy and ragged-red fibre syndrome and axial multiple lipomas [10].

In the literature, involvement of adipose-derived stromal cells (ADSCs) in the development of lipomas has been proposed [11–14]. However, the involvement of ADSCs in the impaired adipose tissue expandability is only beginning to be explored. Dysregulation of ADSC adipogenesis can lead to ectopic adipose tissue formation, as in the case of lipoma [15]. The presence of ADSCs in lipomas with a differentiation potential similar to that of regular ADSCs has been shown in previous studies [11, 12, 16, 17]. Potentially, ADSCs from useless tissue may be an attractive cell source for the application of regenerative medicine [18]. The cells residing in fat stroma revealed a wide range of functional properties *in vivo* as well as differentiation potential *in vitro*. One of the significant practical factors supporting the therapeutic use is the potential to readily obtain an autologous preparation of these cells for injection within the time frame of 1–2 h [19]. Furthermore, it has been supported by many studies that paracrine effects play dominant roles in the therapeutic function of ADSCs. Interestingly, ADSCs can be reprogrammed to induce pluripotent stem cells more efficiently than other cell types. They are immunoprivileged cells and secrete immunomodulatory, angiogenic, anti-apoptotic and haematopoietic factors that facilitate tissue repair [15].

Thus, stem cell-based therapy hold promise for treating acquired and genetic diseases and has been utilized in animal models and human clinical trials, such as wound healing, tissue repair, cardiovascular disorders [19], congenital muscular dystrophy [18] or lower genito-urinary dysfunction [20].

Thus, the aim of this study was twofold: (1) to isolate and characterise ADSCs from patients diagnosed with MSL type I or II and (2) to review the literature and to evaluate the approaches that can explain the disorder at the level of ADSCs.

Patients and Methods

Patients diagnosed with either MSL type I or II and whole-body involvement were included. Informed written consent for molecular analysis was obtained from all patients, and the Institutional Review Board approved the study [EKBB (Ethics Committee from

Basel) reference No. 78/07]. All human experiments were conducted in accordance with the Declaration of Helsinki (1964). The surgical procedure was performed as described previously [21]. Briefly, preoperative markings were performed with the patient in the standing position. Then, 2–3 access incisions of <1 cm were made at the periphery of the lesions. Infiltration of tumescent solution consisting of Ringer's lactate supplemented with adrenaline (1:1,000,000) and lidocaine (0.075%) was performed. For liposuction, the PAL[®] LipoSculptor[™] (MicroAire) was used. An electric speed motor generates a reciprocating motion at 4,000 cycles/min (forward and backward) in the cannula and produces a 2-mm excursion at the tip [21].

Isolation of ADSCs

Samples of fresh lipo-aspirates were enzymatically digested in 0.075% collagenase type II (355 U/mg; Worthington, Lakewood, N.J., USA) for 60–90 min at 37°C on an orbital shaker. After centrifugation at 300 g for 10 min, the lipid-rich layer was discarded and the resulting pellet washed with complete medium (CM), consisting of α -MEM supplemented with 10% fetal bovine serum (FBS), 1% HEPES, 1% sodium pyruvate and 1% penicillin-streptomycin-glutamine ($\times 100$) solution (all from Gibco, www.invitrogen.com) and filtered with a 100- μ m strainer (BD Falcon; BD Biosciences, San Diego, Calif., USA, <http://www.bdbiosciences.com>). Lysis of remaining erythrocytes was achieved by incubation for 10 min at 4°C in ammonium chloride solution (Stemcell Technologies, Grenoble, France). Finally, the solution was filtered with a 70- μ m strainer and the cells resuspended in CM. Total cell numbers were assessed by staining with crystal violet (Sigma) and counting of the nucleated cells in a Neubauer chamber.

Cell Characterisation

Freshly isolated ADSCs were characterised by fluorescence-activated cell-sorting (FACS) analysis. The cells were incubated for 30 min at 4°C with fluorochrome-conjugated antibodies against the indicated antibodies or an isotype control. Antibodies used were: CD31-fluorescein isothiocyanate (FITC) and CD34-APC (endothelial markers), CD45-PE (panhaematopoietic marker), CD73-APC (all from BD Biosciences) and CD105-FITC (AbD Serotec, Oxford, UK; mesenchymal markers). Cells were washed and resuspended in FACS buffer (PBS, 0.5% human serum albumin, 0.5 mM EDTA) and analysed with a FACSCalibur flow cytometer (BD Biosciences USA). The data were analysed using FlowJo software v. 10.0.6 (Tree Star Inc., Ashland, Oreg., USA) and expressed as the percentage of positive cells determined by flow cytometry.

Colony-Forming Efficiency Assay

The number of clonogenic cells was evaluated by the colony-forming efficiency assay as described previously [22]. Briefly, cells were plated at clonal density (9 cells/cm²) and cultured in α -MEM (Gibco) with 10% FBS and 5 ng/ml fibroblast growth factor (FGF)-2. After 2 weeks, cells were washed with PBS, fixed with 3.7% formaldehyde in PBS, stained with crystal violet (Sigma) for 10 min and washed with tap water, and then colonies were counted. In order to exclude possible sporadic macrophage aggregates, only colonies that were macroscopically visible and composed of fibroblastic cells were considered. All determinations were performed in triplicate [22].

Fig. 1. Representative figure of a 37-year-old female patient with MSL type II before lipoplasty of the upper arms.

Adipogenic Differentiation

To further demonstrate the power of adipogenic plasticity of ADSCs, cells were differentiated toward the adipocyte lineage, as previously reported [23, 24]. Briefly, stromal cells were isolated using 0.15% collagenase digestion as well as washing and centrifugation cycles. Then, adipocyte differentiation was induced after seeding 2×10^4 cells/well on 6-well tissue culture plastic dishes and growing them with Dulbecco's modified Eagle's medium [DMEM ($\times 1$)] supplemented with 10% FBS, 1% HEPES, 1% sodium pyruvate and 1% penicillin-streptomycin-glutamine ($\times 100$; all from Gibco) until 95% confluence. To induce adipogenic differentiation, the 10% DMEM solution was supplemented with 10 $\mu\text{g}/\text{ml}$ insulin, 1 μM dexamethasone, 100 μM indomethacin and 0.5 mM 3-isobutyl-1-methylxanthine for the first 72 h and then with 10 $\mu\text{g}/\text{ml}$ insulin for the next 24 h [25]. Three differentiation cycles of 96 h were performed as previously described. The cells were washed once with PBS, fixed with 4% formaldehyde, stained with oil red O 0.3% (Sigma) and photographed with an Olympus IX50 microscope (Olympus Corporation, Tokyo, Japan). The staining was solubilized with isopropanol (Emsure; Merck Millipore, Billerica, Mass., USA) and quantified by measuring absorbance at 500 nm with a Synergy H1 hybrid multimode spectrofluorometer. The differentiated ADSCs were compared to BLK (= blank, pure isopropanol) and non-differentiated ADSCs, which were cultured in CM during the same time period.

Data are presented as means $\pm$ SD (GraphPad Prism version 5.00 for Mac OS X; GraphPad Software, San Diego, Calif., USA, www.graphpad.com).

Literature Search Strategy

A literature review was performed from 1982 to 2015 using the US National Institutes of Health's PubMed database. The reference lists of all selected papers were further reviewed for potentially relevant analysis. Eligible reports were full papers written in English language reporting on the pathophysiology of lipoma and ADSCs.

Article Selection

Combinations of the following key words were used: 'lipoma' and 'stem cells'. Totally, 36 articles were identified. Of those, 30

were included in the review: there were 12 case reports [1, 9–11, 26–34], 12 reports on basic science or animal studies [4, 12, 13, 35–44], 2 case series [14, 45] and 4 systematic reviews [13, 38, 46, 47]. Totally, more than 60 patients were analysed; 25 articles were studied as a full-text version.

Results

Two male and 1 female, European-descent patients aged between 37 and 55 years (mean 49 ± 10 years) were identified and diagnosed with MSL. Two patients were diagnosed with MSL type II (fig. 1), whereas 1 patient was diagnosed with MSL type I.

Characterization of ADSCs and Colony-Forming Efficiency Assay

Taken all the results pooled together, flow-cytometric analyses of the ADSCs revealed high levels of CD34, CD45 and CD73, but low levels of CD31 and CD105 were detected (fig. 2). The results of the patients' ADSCs compared to regular ADSCs from historical normal patients are shown in table 1. The resulting mean of the colony-forming efficiency assay, which is an approximation to the frequency of progenitor cells within the adipose tissue in isolated ADSCs, was $14.3 \pm 5\%$.

Adipogenic Differentiation

We confirmed adipogenesis 2 weeks after induction by oil red O staining, showing intracellular lipid droplets of variable sizes (fig. 3). Semi-quantitative analysis showed a high amount of lipid droplets in the differentiated ADSCs (10.2 ± 0.4 OD).

Fig. 2. Representative flow-cytometric analysis of undifferentiated lipoma-derived multipotent mesenchymal stromal cells derived from an individual donor were stained with monoclonal antibodies directed against either CD34, CD31, CD45, CD73 or CD105 and coupled to FITC. An isotype-matched monoclonal antibody served as a control.

Discussion

The clinical expression of MSL is sex related: in type I MSL, lipomatous tissue is located around the neck, upper trunk and arms. This type occurs in men; patients are usually underweight and uninvolved subcutaneous tissue is often reduced or even atrophic [48]. Type II MSL affects both men and women and is characterized by adipose tissue accumulation in the upper back, deltoid regions, up-

per arms, hips and thigh regions [48]. It is suggested that there is a causal relationship between higher alcohol consumption and MSL expression [48], which we observed in 2 patients from our case series. Furthermore, the accumulation of subcutaneous fat leads to changes in the subcutaneous/visceral fat content ratio, and this may reduce insulin resistance analogous to the action of antidiabetic drugs like peroxisome-proliferator-activated γ -receptor agonists (thiazolidinediones) [48]. It remains speculative

Fig. 3. Representative photomicrographs from controls (undifferentiated ADSCs) and ADSCs under adipogenic conditions (a). Adipogenic cultures (undifferentiated ADSCs) (b) were stained with oil red O 14 days after induction (c).

Table 1. Characterisation of ADSCs from patients diagnosed with MSL

Patient	Sex/age, years	Diagnosis	Donor site	CFU-f, %	CD31, %	CD34, %	CD45, %	CD73, %	CD105, %
1	M/55	MSL type I History of alcohol consumption Hepatic disease Hypertriglyceridemia	Lower back and neck	11.9±1.0	10.6	66.0	39.1	53.3	5.2
2	M/54	MSL type II History of alcohol consumption Hyperuricemia	Upper arms	10.2±0.5	7.4	63.5	48.6	43.0	5.6
3	F/37	MSL type II Diabetes mellitus II Hypertriglyceridemia Hyperuricemia Kidney disease	Upper arms	20.8±0.3	NA	NA	NA	NA	NA
References 57, 59, 67–69				3.125±2.1–13±5	21.8±10.8	60.0±11.5	17.6±7.7	25.0±6.2	4.9±3.5
Data are presented as means ± SD. NA = Not available.									

whether hyperinsulinemia and alcohol consumption have a synergistic impact on the extent of the disease. Nevertheless, it is suggested that, in addition to the cessation of alcohol consumption, the reduction in blood glucose and lipid concentrations by medication may also assist in resolving the accumulated fat of type I MSL in patients with non-insulin-dependent diabetes mellitus [49].

MSL, a mitochondrial disorder derived from vestigial brown fat, is characterised by the development of axial lipoma [10, 50]. Complete removal is almost impossible and recurrences inevitable, among others due to the underlying primary metabolic defect, lack of encapsulation, the large size and anatomic distribution of the lesions [21, 51]. Furthermore, lipoma-derived stem cells have been suggested to play an important role in lipoma recurrence after lipoma liposuction [52].

In our study, molecular analysis of diseased adipose tissue of whole-body-affected patients revealed a high ADSC number in the colony-forming efficiency assay. Furthermore, strong adipogenic plasticity has been observed *in vitro*. In addition, ADSCs showed a high expression of CD34, CD45 and CD73, and low levels of CD31 and CD105, respectively. Although this cell characterisation is not in line with the minimal set of standard criteria of multipotent mesenchymal stromal cells [53], ADSCs may have a different cell phenotype [15]. Potentially, the positivity of CD34 and CD31 might be characteristic for these cells, given the tumoural feature of ADSCs. This is in line with the recent literature, which suggested that CD34+ lipoma cells showed higher vasculogenic properties and increased resistance in aging [14]. The potential roles of the CD34 family include increasing proliferation (replicative capacity) and blocking differentiation of stem or progenitor cells that therefore maintain their undifferentiated progenitor/stem cell phenotype (immaturity or stemness of the cells) [14]. However, a limitation of our study is the lack of ADSCs obtained from subcutaneous adipose tissue from the same donor to really compare biological characteristics of ADSCs from lipoma.

ADSCs from lipoma tissue have been shown to transform into various histological characteristics and types such as angiolipoma, lipoma, myolipoma, lipomatous neurofibroma, osteolipoma or osteochondrolipoma [1, 11, 29–32]. In 2007, Lin et al. [12] described for the first time putative ADSCs from lipoma tissue with characteristics similar to regular ADSCs but with no evidence of malignant transformation. ADSCs from lipomas showed a significantly higher proliferative capacity than regular ADSCs with increased numbers of Ki67+/CD34+ and the ability to differentiate into fat, bone or cartilage [23]. This

‘multipotency’ may be derived from different progenitor cell types within the lipoma.

Furthermore, it was observed that CD34+ ADSCs aggregate around small adipocytes in lipoma tissue, also called adipogenic/angiogenic cell cluster, which is suggestive of adipocyte neogenesis [41]. Interestingly, it has also been proposed that brown adipocyte precursors arise in close association with vessel walls [27]. Lastly, it has been suggested that there is a close histogenetic link between lipomatous and angiomyofibromatous processes induced by perivascular stem cells [28, 45].

Our results, which confirm a high turnover of adipocytes, are in line with previous studies [14, 23, 54, 55]. The colony-forming unit-fibroblast (CFU-f) assay is a popular method to evaluate stem cell capacity [56]. In general, the frequency of stromal progenitors ranges from 1 to 10% of the total nucleated cell population. In our study, however, the CFU-f assay showed a higher frequency ($14.3 \pm 5\%$) in MSL patients [57]. Importantly, it is known that FGF improves ADSC proliferation [58]. This was confirmed in a recent study [59] where CFU-f was determined by plating stromal vascular fraction cells cultured in CM containing FGF, showing a high proliferation rate ($>10\%$) [59].

It has been suggested that high mobility group (HMG) proteins act as architectural factors and coordinate cell proliferation and differentiation in adult adipose tissue [36, 37, 46]. Research revealed that the HMGA2 gene is frequently disrupted in human solid tumours of a differentiated mesenchymal origin such as lipoma [46]. Furthermore, it has been postulated that PPAP2B, a member of the lipid phosphate phosphatases family, plays a role in human adipogenesis [36]. Finally, pRB, a product of the human RB-1 gene, might play a key role in adipogenesis [39].

The cumulative population-doubling level of lipoma ADSCs is significantly higher than that of regular ADSCs [23]. Thus, they possess a potent proliferation potential in less time than regular ADSCs [23]. In a study by Qian et al. [55], there were significant differences between regular ADSCs and lipoma ADSCs in the biological characteristics *in vitro* in terms of *in vitro* biological features. In this study, regular ADSCs showed a significantly lower proliferation capacity than ADSCs from lipoma, as evidenced by MTS chromatometry (= assay for assessing cell metabolic activity).

Apoptosis represents a process of programmed cell death [60]. It has been proposed that cellular apoptosis is not enhanced in lipoma, suggesting that the enlargement of lipoma tissue may be due to a positive balance of adipocyte turnover (accelerated adipogenesis combined with non-enhanced apoptosis) [41]. In a recent study by

Zavan et al. [14], increased expression of genes associated with the increased negative regulation of the apoptotic event and down-regulation of apoptotic markers have been found in lipoma tissue. Although adipogenesis is accelerated in lipoma, lipoma tissue escapes from hypoxia and does not show macrophage infiltration, subsequent inflammatory changes or enhanced apoptosis [41]. It has been postulated that angiogenesis may be successfully increasing alongside adipogenesis, explaining the reason for the maintenance of oxygen pressure in lipoma [41]. Indeed, our results suggest increased adipogenesis and neoangiogenesis, confirmed by a high expression of CD34+ in our cell population. CD34 has been suggested to be an excellent marker of neoangiogenesis; it has been used to highlight the microvasculature vessel density as a direct marker of the degree of neoangiogenesis [61]. It is a highly glycosylated transmembrane cell surface glycoprotein expressed by haematopoietic stem and progenitor cells and presents on the luminal cell membrane of quiescent endothelial cells of small blood vessel and lymphatics [62]. In lipoma tissue, the persistence of CD34 positivity is associated with increased replicative capacity and maintenance of cell immaturity [14]. Importantly, CD34+ cells regulate stromal collagen content and help to activate local fibroblasts through increased proliferative capacity, stimulation of growth factors and extracellular matrix protein [14].

It has been shown that passaged human ADSCs reduce their expression of surface histocompatibility antigens and no longer stimulate a mixed lymphocyte reaction when co-cultured with allogeneic peripheral blood monocytes [13]. Potentially, transplanted allogeneic ADSCs will not elicit a robust immune response and subsequent rejection [13]. Thus, they may have potential therapeutic use as allogeneic products for tissue engineering and for gene therapy upon genetic modification as well [40]. That being said, genetic stability of these cells is of paramount importance [40]. Interestingly, it has also been shown that ADSCs are deficient in gap-junctional intercellular communication [23]. Moreover, it has been suggested that the β_3 -adrenergic receptor is the functionally relevant adrenergic receptor subtype in brown adipocytes and that its stimulation by noradrenaline modulates the expression of genes, such as uncoupling protein-1 and inducible nitric oxide synthase, involved in fat cell proliferation and differentiation [63]. The failure of noradrenaline to induce nitric oxide synthase production seems to be involved in the dysregulated proliferation and differentiation (i.e. mitochondrial biogenesis) of ADSCs [63].

Thus, undifferentiated ADSCs might be potential major modulators of MSL. Next to the obvious application of ADSCs as precursors of differentiated cells for cell replacement, the unique immune biology and secretome of ADSCs are increasingly appreciated for their therapeutic potential [15]. As ADSCs are immunoprivileged due to the lack of expression of class II major histocompatibility complex and co-stimulatory molecules on the cell surface, this potentially allows allogeneic transplantation of ADSCs into immunocompetent recipients with minimal immune reaction in the host [64]. Furthermore, ADSCs also secrete an array of soluble factors that promote tissue regeneration at the injury site via a paracrine mechanism. The secretome includes angiogenic factors, anti-apoptotic factors, haematopoietic factors and hepatocyte growth factor. It is now suggested that the effects of ADSC therapy in vivo are largely attributable to the paracrine and immunomodulatory effects of ADSCs rather than the cell replacement per se [65]. Interestingly, ADSCs are also ideal for reprogramming into induced pluripotent stem cells [15]. It has been shown that reprogramming somatic cells from patients with mitochondrial DNA disorders can yield pluripotent stem cells with varying burdens of heteroplasmy, which might be useful in the study and treatment of mitochondrial diseases such as MSL [66].

Conclusions

The properties and knowledge of ADSCs from lipoma bear the hope of making useless tissue useful in the application of tissue engineering and regenerative medicine. Eventually, a better understanding of ADSC features from lipoma (multipotency of clonal cells, serial transplantation assays, in vivo studies, migration, cytokine profiles, proliferation ability, dual labelling and complete FACS analysis) together with the determination of co-expression markers on the same cell by immunohistochemistry of the lipoma tissue compared to a formal control group may be useful to regulate their activities and to slow their metabolism at the source of the MSL.

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Disclosure Statement

The authors have no conflict of interest.

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