Cellular and molecular features of lipoma tissue: comparison with normal adipose tissue


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Summary

Background Involvement of adipose-derived stem/progenitor/stromal cells (ASCs) in the development of lipomas has been suggested, but the pathogenesis and pathophysioloogy of this tumour remain unclear.

Objectives To analyse cellular and transcriptional characteristics of lipoma tissue compared with normal adipose tissue, further to delineate differentiating features.

Methods For lipoma or normal adipose tissues, we used a new whole-mount staining enabling three-dimensional imaging of nonfixed and nonfrozen adipose tissue. Immunohistochemistry and real-time polymerase chain reaction for obesity-related genes were performed as well as comparative assay of the proliferative and adipogenic capacity of ASCs.

Results A large number of small adipocytes surrounded by CD34+ ASCs and increased numbers of Ki67+ CD34+ ASCs indicated enhanced adipogenesis in lipoma compared with normal adipose tissue. In contrast, cellular apoptosis was 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 nonenhanced apoptosis). Leptin mRNA was upregulated in lipoma, while adiponectin, tumour necrosis factor-α and glucose transporter 1 mRNA were downregulated and there were no apparent changes in hypoxia-inducible factor 1α, peroxisome proliferator-activated receptor-γ and plasminogen activator inhibitor-1. These results suggested dysfunction of lipoma adipocytes similar to that in obesity, but indicated that lipoma tissue lacked several obesity-related phenomena such as ischaemia (hypoxia), macrophage infiltration, inflammatory reactions and enhanced glycolysis. ASCs from lipoma and normal adipose tissue showed similar proliferative and adipogenic capacity.

Conclusions Our findings revealed that lipoma tissue shows a positive balance of adipocyte turnover involving proliferating ASCs and several transcriptional differences from adipose tissue enlargement in obesity.

Lipoma is a common benign neoplasm, which frequently arises in the subcutaneous tissues, is most commonly located in the trunk and proximal limbs, and is demarcated from surrounding adipose tissue by a thin fibrous capsule. While lipomas are usually treated when small in size, they can grow larger than 10 cm and can weigh over 1 kg. Although there are reports in the literature of proliferative activity and chromosomal aberrations of lipoma adipocytes, details of the cellular and molecular phenomena underlying the pathogenesis of this tumour remain unclear.

Adipocyte progenitor cells are found in normal adipose tissue, and the turnover rate of normal adipocytes is very slow. Adipose progenitor cells have been shown to have the capacity to differentiate into multiple lineages and are referred to as adipose-derived stem/progenitor/stromal cells (ASCs). Human ASCs have been determined to be CD34+ in vitro and in vivo, although they tend to lose CD34 expression with culture time. Spindle cell lipoma is also reported to be CD34+; this type of lipoma consists of an intricate mixture of adipocytes and uniform spindle cells.

Although ASCs can be isolated from human lipoma tissue, detailed analysis of lipoma tissue at the cellular and molecular levels has rarely been performed. Conventional histological analysis of adipose tissue with frozen or paraffin-embedded sections has limitations that prevent accurate, detailed examination: freezing or fixation easily disrupts fragile adipocytes.
and surrounding structures, and sectioning of the tissue leads to leakage of lipid contained in adipocytes; these artifactual deformations deform the original structure of adipose tissue. In this study, we employed a whole-mount immunohistological method that enables three-dimensional visualization of living adipose tissue stained with triple fluorescence.\textsuperscript{17,18} In addition, we analysed cellular and transcriptional characteristics of lipoma tissue and normal adipose tissue, which may provide new insights into the pathogenesis and pathophysiology of lipomas.

**Materials and methods**

**Clinical data**

Intact adipose tissue alone (n = 2), lipoma tissue alone (n = 2), or both (n = 3) were obtained from seven nonobese patients (mean ± SEM body mass index 22.3 ± 1.1 kg m\(^{-2}\)) who underwent plastic surgery or tumour resection. The patients were four men and three women aged 47 ± 5 years (mean ± SEM) (Table 1). Informed consent was obtained from each patient using a protocol approved by the institutional review board.

**Whole-mount staining**

Visualization of nonfixed and nonsectioned adipose tissues was performed using a whole-mount staining as previously described.\textsuperscript{17,18} Briefly, adipose tissue samples were minced into 3-mm pieces and incubated with the following reagents for 30 min at 37 °C: BODIPY 558/568 (5 µg mL\(^{-1}\); Molecular Probes, Eugene, OR, U.S.A.) was used to stain adipocytes, Alexa Fluor 488-conjugated isoelectin GS-IB4 (lectin) (5 µg mL\(^{-1}\); Molecular Probes) to stain endothelial cells, and Hoechst 33342 (5 µg mL\(^{-1}\); Dojindo, Kumamoto, Japan) to stain nuclei. For CD34 immunostaining, the tissue sample was fixed in 4% paraformaldehyde for 30 min and incubated with Alexa Fluor 488-conjugated goat antimouse IgG and Alexa Fluor 568-conjugated goat antirabbit IgG (both dilution 1 : 200; Molecular Probes), together with lectin and Hoechst 33342. Each stained sample was directly observed with a confocal microscope system (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). Thirty serial images were obtained at 1-µm intervals and the acquired images were processed to produce a surface-rendered 30-µm thick three-dimensional image.

**Immunohistochemistry**

Harvested adipose tissues were zinc-fixed (Zinc Fixative; BD Biosciences, San Diego, CA, U.S.A.) and paraffin-embedded. We prepared 6-µm thick sections and performed immunostaining using the following primary antibodies: mouse antihuman CD34 (clone QBEnd 10, dilution 1 : 500; Dako) and rabbit antihuman Ki67 (clone SP6, dilution 1 : 200; Thermo Fisher Scientific, Fremont, CA, U.S.A.) or mouse antihuman CD68 (clone KP1, dilution 1 : 100; Dako). Isotypic antibody was used as a negative control for each staining. For visualization with diaminobenzidine, peroxidase-conjugated secondary antibodies appropriate for each primary antibody (Nichirei Biosciences, Tokyo, Japan) were used. Nuclei were counterstained with haematoxylin. For double-fluorescence staining, Alexa Fluor 488-conjugated goat antimouse IgG and Alexa Fluor 568-conjugated goat antirabbit IgG (both dilution 1 : 200; Molecular Probes) were used as secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole. To detect apoptosis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) staining was performed using an in situ Cell Death detection kit (Roche Diagnostics, Mannheim, Germany).

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from the samples using an RNAasy Mini Kit (Quigien, Hilden, Germany), followed by reverse transcription. Real-time polymerase chain reaction (PCR) was performed with the ABI 7700 sequence detection system and a SYBR Green PCR master mix (both from Applied Biosystems, Foster City, CA, U.S.A.); the primer sequences used are shown in Table 2. Expression levels were calculated by the comparative C\(_t\) method using glyceraldehyde-3-phosphate dehydrogenase as an endogenous reference gene.

**Stromal cell isolation and culture**

Adipose tissue samples were minced into 2–3-mm pieces and digested on a shaker in phosphate-buffered saline containing 0.075% collagenase (Wako Chemicals, Osaka, Japan) for 30 min at 37 °C. Mature adipocytes and connective tissue were separated from pellets by centrifugation (800 g, 10 min). The stromal vascular fraction cells containing ASCs were resuspended, filtered through 100-µm mesh, plated at a density of 2 × 10\(^5\) nucleated cells/60-mm dish, and cultured...
at 37 °C in an atmosphere of 5% CO₂ in humid air. Primary cells, defined as 'passage 0', were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) and cells other than ASCs (such as endothelial cells and leukocytes) were almost excluded by the adherent culture for 1 week. The medium was replaced every 3 days and the cells were subcultured every week by trypsinization.

Adipogenic differentiation assay

The stromal vascular fraction cells at passage 1, most of which are composed of ASCs, were incubated for 21 days in DMEM containing 10% FBS, 0.5 mmol L⁻¹ isobutyl-methylxanthine, 1 μmol L⁻¹ dexamethasone, 10 μmol L⁻¹ insulin and 200 μmol L⁻¹ indomethacin. Adipogenic differentiation was visualized with Oil Red O staining. For quantitative analysis of lipid droplets, Nile Red fluorescence (AdipoRed; Cambrex, Walkersville, MD, U.S.A.) was measured with excitation at 485 nm and emission at 535 nm.

Statistical analysis

Results were expressed as mean ± SEM. Comparisons between two groups were performed with Welch’s t-test. P < 0.05 was considered significant.

Results

Whole-mount histology of living tissues

Whole-mount histology showed nonruptured adipocytes with capillaries located alongside the adipocytes, forming a well-organized network in both lipoma and normal adipose tissue (Fig. 1a; adipocytes in green and capillaries in red). Capillaries and vessels were abundant in both tissues, and no apparent difference in vascular density was observed (Fig. 1a). Small adipocytes (< 50 μm in diameter) were detected in both tissues; the proportion of these small adipocytes was significantly higher in lipoma tissue (*P < 0.05). Each symbol corresponds to a sample donor shown in Table 1. Bars show mean ± SEM.

(a) M...
tissue, forming a cell cluster suggestive of adipocyte neogenesis (Fig. 2).

**Histology of fixed sections**

Using fixed tissue sections, we examined cell proliferation, apoptosis and macrophage infiltration. Immunohistochemical staining for Ki67 indicated that the proportion of Ki67+ proliferating cells was significantly higher in lipoma tissue (6.90 ± 1.12%) compared with normal adipose tissue (2.41 ± 0.61%) (Fig. 3a,b). Double-fluorescence immunostaining for Ki67 and CD34 revealed that most Ki67+ proliferating cells in lipoma were also positive for CD34 (Fig. 3c). These results suggest that ASCs are the primary proliferating cell population and contribute to enhanced adipogenesis in lipoma.

TUNEL staining indicated that there were no differences in apoptotic cell number between lipoma and normal adipose tissue (Fig. 4a,b). Immunohistochemical staining for CD68 showed that CD68+ macrophages were rare in both lipoma and normal adipose tissue; crown-like structures, a feature of adipocyte phagocytosis by infiltrating macrophages frequently seen in obese adipose tissue,19–21 was not observed (data not shown).

**Assays for expression of obesity-related genes**

Expression of selected obesity-related genes was examined with real-time PCR (Fig. 5). Leptin expression was higher and adiponectin expression was lower in lipoma tissue compared with normal adipose tissue in all three patients from whom both tissues were harvested; this expression pattern is a characteristic finding in obesity.22,23 Tumour necrosis factor (TNF)-α, hypoxia-inducible factor 1α (HIF1α), plasminogen activator inhibitor-1 (PAI-1) and glucose transporter 1 (GLUT1) are known to be upregulated in obesity.22,23 However, in our samples no apparent difference in expression of HIF1α and PAI-1 was detected between lipoma and normal adipose tissue, while...
expression of TNF-α and GLUT1 were downregulated in lipoma tissue of all three patients. Expression of peroxisome proliferator-activated receptor-γ (PPARγ) in lipoma tissue was not distinctly different from that in normal adipose tissue.

**Assays for adipose progenitor cell functions**

ASCs were isolated and cultured from lipoma and normal adipose tissue. Adipogenesis was easily induced in ASCs from both tissues, and there was no difference in the capacity for adipogenic differentiation (Fig. 6a). Long-term adherent culture showed that both cell populations possess similar expansion potentials at least up to passage 10 (Fig. 6b). There were no morphological differences between the cells from lipoma and normal adipose tissue after long-term culture (Fig. 6c). Thus, we did not detect any functional differences between ASCs from lipoma and normal adipose tissue in vitro assessments, although they apparently behave differently in vivo.

**Discussion**

Our findings indicate that ASCs in lipoma tissue are actively proliferating and contribute to enhanced adipogenesis. A cell...
aggregation formed with a small adipocyte surrounded by CD34+ ASCs seen in lipoma tissue is a characteristic finding suggesting adipogenesis, which was also seen in diabetic obese mice and is called an adipogenic angiogenic cell cluster.17 In these cell clusters, it is likely that ASCs are proliferating and supporting the small differentiating adipocytes.

Our results also showed that apoptotic adipocyte number is comparable in lipoma and normal adipose tissue, suggesting that the enlargement of lipoma tissue may be due to the positively preserved balance of adipocyte turnover (accelerated adipogenesis with nonenhanced apoptosis). A previous study demonstrated proliferation of cells alongside vessels in lipoma, the proliferating cells seem to be ASCs, based on previous demonstration of a perivascular localization for ASCs6,11,24 as well as in this study. In spindle cell lipoma, an intricate mixture of adipocytes and uniform spindle cells14,15 which were strongly positive for CD34, were observed;12,13 the CD34+ ASCs may be expanding at a greater rate than that seen in normal adipogenesis. Our study showed no apparent differences in properties or functional status of ASCs derived from lipoma and normal adipose tissue in vivo, even though they behave differently in vivo. This finding suggests that increased activity of lipoma ASCs may be highly dependent on genetic or microenvironmental factors. PPARγ plays a central role in adipocyte differentiation6,25 and activation of PPARγ signalling induces terminal differentiation of human liposarcoma cells.26–27 PPARγ expression did not differ between lipoma and normal adipose tissue, suggesting involvement of other mechanisms in lipoma, such as injury-associated factors.18

It is of great interest that our lipoma tissues showed some features in common with and some distinct from obesity. Similar to obese adipocytes, lipoma-derived adipocytes showed upregulation of leptin and downregulation of adiponectin; this suggested impaired endocrine function of lipoma adipocytes. However, in contrast to obese adipocytes, lipoma adipocytes lacked macrophage infiltrations, upregulation of TNF-α and HIF1α, and increased glycolysis (GLUT1 and PAI-1). Recent studies indicated that adipose tissue in obesity is hypoxic, and this hypoxia is a basis for the dysregulation of tissue function in obesity.22,23 Hypoxia in obese adipose tissue induces HIF1α upregulation, activates glycolytic processes, and induces adipocyte apoptosis/necrosis, which further recruits macrophages and enhances inflammatory reactions.19 Infiltrated macrophages play a crucial role in the development of obesity-related insulin resistance21 and upregulation of inflammatory cytokines such as TNF-α.20 Our results showed that hypoxia-related genes, such as HIF1α, GLUT1 and PAI-1, were not upregulated in lipoma samples, suggesting that normal oxygen pressure in lipoma tissue is preserved. Although adipogenesis is accelerated in lipoma, lipoma tissue escapes from hypoxia and does not show enhanced apoptosis, macrophage infiltration, or subsequent inflammatory changes. A potential explanation for this finding is that angiogenesis may be successfully increasing alongside adipogenesis, and this may be the reason for maintenance of oxygen pressure in lipoma. We previously reported a case of lipedema, in which increased adipogenesis and proliferating ASCs were observed in the tissue, similar to lipoma; however, lipedema tissue also showed hypoxia-associated features such as adipocyte death and macrophage recruitment.29

In conclusion, comparative analyses of lipoma and normal adipose tissue showed enhanced adipogenesis and nonenhanced adipocyte apoptosis in lipoma tissue, involvement of CD34+/lectin− ASCs in the adipogenesis/angiogenesis process, and adipocyte dysfunction such as upregulation of leptin and downregulation of adiponectin. We also found that lipoma tissue lacked several obesity-related phenomena such as ischaemia (hypoxia), macrophage infiltration, inflammatory reactions and enhanced glycolysis. Functional differences of
tissue progenitor cells were not detected, suggesting that the increased adipogenesis/angiogenesis may be dependent on microenvironmental factors of lipoma tissue, although further studies will be needed to elucidate detailed mechanisms underlying this phenomenon.

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References