II. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
副島英伸，城圭一郎，中尾光善	23章エピジェネティクスとヒト疾患	D. アリス・T. ジェニュワイン・D. ラインバーグ共編，堀越正美監訳.	エピジェネティクス	培風館	東京	2010	505-528
副島英伸	第ＸＩＶ章先天異常･奇形　ベックウィズ･ヴィーデマン症候群	井村裕夫総編集，福井次矢・辻省次編集	症候群ハンドブック	中山書店	東京	2011	P679
副島英伸	第ＸＩＶ章先天異常・奇形　シルバー･ラッセル症候群	井村裕夫総編集，福井次矢・辻省次編集	症候群ハンドブック	中山書店	東京	2011	P685

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamamoto S, Toyama D, Yatsuki H, Higashimoto K, Soejima H, Isoyama K.	Acute megakaryocytic leukemia (AMKL,FAB;M7) with Beckwith-Wiedemann syndrome	*Pediatr Blood Cancer*	55(4)	733-735	2010
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Yamazawa K#,Nakabayashi K#, Kagami M, Sato T, Saitoh S, Horikawa R, Hizuka N, Ogata T.	Parthenogenetic chimaerism/mosaicism with a Silver-Russell Syndrome-like phenot	*J Med Genet*	47(11)	782-785	2010
秦健一郎	胎児発育とゲノムインプリンティング	*HORMONE FRONTIER IN GYNECOLOGY*	17	43-48	2010
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Nakanishi M, Hayakawa K, Nakabayashi K, Hata K, Shiota K, Tanaka S.	Trophoblast-specific DNA methylation occurs after the segregation of trophectoderm and inner cell mass in mouse periimplantation embryo.	*Epigenetics*	7(2)	173 - 182	2012
Kobayashi H, Sakurai T, Sato S, Nakabayashi K, Hata K, Kono T.	Imprinted DNA methylation reprogramming during early mouse embryogenesis at the *Gpr1-Zdbf2* locus is linked to long cis-intergenic transcription.	FEBS Letters			in press

III. 研究成果の刊行物・別冊

ベックウィズ・ヴィーデマン症候群

Beckwith-Wiedemann syndrome (BMS)

【ICD-10】Q87.3
【OMIM】#130650
【特記事項】厚生労働省難治性疾患克服研究事業 研究奨励分野の対象疾患

■疫学　国内推定有病者数／200人以上
男女比／1:1

■発症に関わる遺伝子　*KVDMR1*（11p15.5），*CDKN1C*（11p15.5），*KCNQ1OT1*（11p15.5），*H19-DMR*（11p15.5），*IGF2*（11p15.5），*H19*（11p15.5）

■診断　診断基準は主症状3つ以上，または主症状2つと副症状1つ以上とされているが，確定的な基準はない．

主症状：腹壁欠損（臍帯ヘルニア，腹直筋解離，臍ヘルニア），巨舌，過成長（>97パーセンタイル），耳垂の線状溝・耳輪後縁の小窩，腹腔内臓器腫大，胎児性腫瘍，片側肥大，副腎皮質細胞の腫大（びまん性，両側性），腎奇形，家族歴，口蓋裂

副症状：妊娠中の羊水過多・胎盤腫大・臍帯肥厚・早産，新生児期低血糖，火焔状母斑，心肥大・心奇形・心筋症，特徴的顔貌，骨年齢亢進

上記の臨床診断に加え，*KVDMR1*の低メチル化あるいは*H19-DMR*の高メチル化や微小欠失，*CDKN1C*変異，11p15.5父性片親性ダイソミーを検出すると確定診断できる．

■治療　低血糖については生後の血糖値モニタリング．臍ヘルニアに対しては外科的根治術．8歳くらいまでは腹部超音波検査で3か月ごとに腫瘍をスクリーニング．4歳までは定期的にαフェトプロテイン（AFP）も測定．過度の巨舌に対しては舌縮小術を，片側肥大による下肢長の左右差（1～2cm以上）に対しては骨端固定術を考慮する．

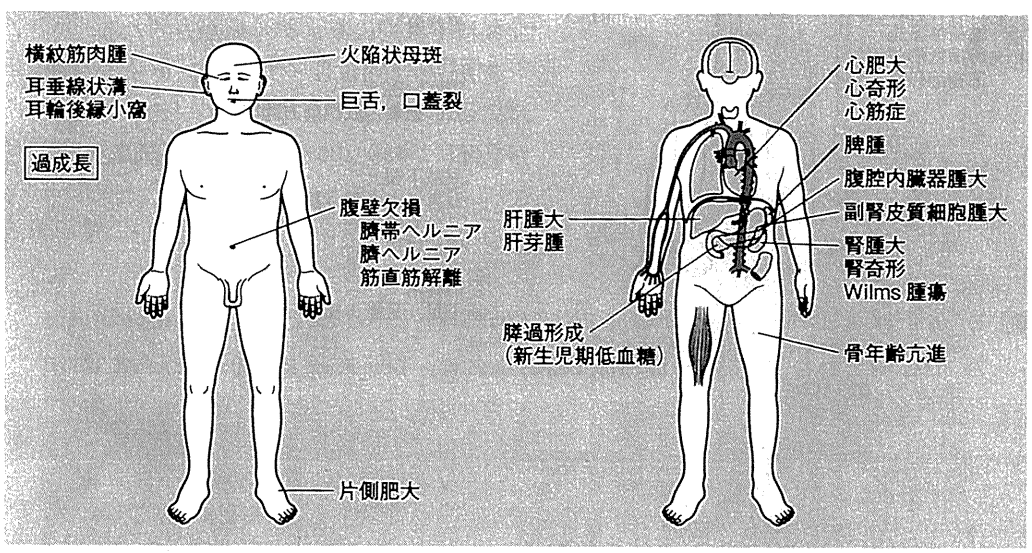
■関連語・同義語　ヴィーデマン・ベックウィズ症候群，exomphalos-macroglossia-gigantism症候群（EMG症候群）

■EBM・診療ガイドライン　厚生労働省難治性疾患克服研究事業平成21年度研究報告書

■関連団体・学会　Beckwith-Wiedemann症候群親の会

■解説　John Bruce Beckwith（1933年生）とHans-Rudolf Wiedemann（1915–2006）が，臍ヘルニア，巨舌，巨躯を伴う症例を1969年に別々に報告した．11p15.5のゲノムインプリンティングの異常で発症し，約7～11%に腫瘍を合併する．診断の項目に記載した遺伝子異常が認められる．　（副島英伸）

■所見



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シルバー・ラッセル症候群　Silver-Russell syndrome (SRS)

【ICD-10】Q87.1
【OMIM】#180860
【特記事項】厚生労働省難治性疾患克服研究事業 研究奨励分野の対象疾患

■疫学　国内推定有病者数／160人以上（実際には非常に多いと推定される）
男女比／1：1

■発症に関わる遺伝子　*H19-DMR*（11p15.5），*IGF2*（11p15.5），*H19*（11p15.5），*CDKN1C*（11p15.5），*KCNQ1OT1*（11p15.5），7番染色体母性ダイソミー（原因遺伝子は未同定）

■診断　子宮内発育遅延，catch-up growthを伴わない低身長，逆三角形の顔貌（前額部突出，小さくとがった下顎，口角下降によるへの字様口唇），身体の左右非対称を主徴とする．他に，第5指内彎，第2・3趾の合趾症，性早熟，尿道下裂，後部尿道弁，鼠径ヘルニアなど多様な症状を呈する．診断基準が提案されている[1,2]．

■治療　発達は原則的に正常だが，最終身長は小児期の身長に比例する．哺乳・摂食障害傾向にあるので，乳児期には適切な食事量を心がける．SGA（small-for-gestational age）性低身長症の1つとして，基準を満たせば成長ホルモン治療を行う．

■関連語・同義語　Russell-Silver症候群，Silver症候群

■EBM・診療ガイドライン　厚生労働省難治性疾患克服研究事業平成21年度研究報告書，日本小児内分泌学会・日本未熟児新生児学会「SGA性低身長症におけるGH治療のガイドライン」「SGA性低身長症におけるGH治療の実施上の注意」

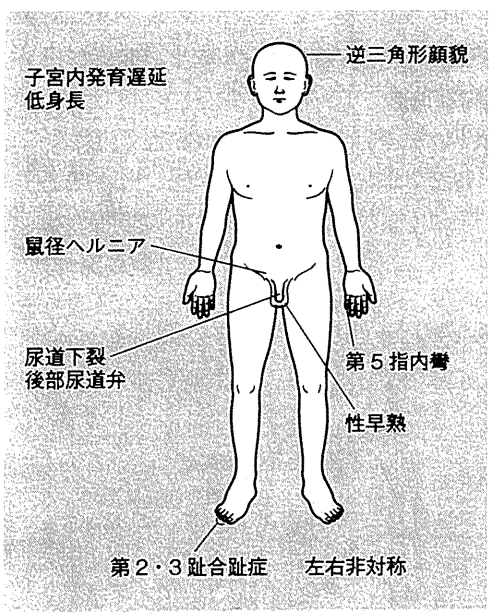
■関連団体・学会　SRS患児・家族の交流を目的としたウェブサイトがある．

■解説　Henry K Silver（1918–1991）が先天性片側低形成，低出生体重，低身長，尿中ゴナドトロピン増加を伴う症例を1953年に，Alexander Russell（1914–2003）が，子宮内発育遅延，上述の特徴的顔貌，左右非対称を伴う症例を1954年に報告した．患児の約10%で7番染色体母性ダイソミーを，約30%で11p15.5インプリンティング領域の*H19-DMR*の低メチル化が認められる．

過成長を示すBeckwith-Wiedemann症候群では逆に高メチル化がみられる．

また，11p15.5インプリンティング領域の母性重複および母性ダイソミーの報告がある．　（副島英伸）

■所見



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Pediatr Blood Cancer 2010;55:733–735

BRIEF REPORT
Acute Megakaryocytic Leukemia (AMKL,FAB;M7) With Beckwith–Wiedemann Syndrome

Shohei Yamamoto, MD,[1,*] Daisuke Toyama, MD,[1] Hitomi Yatsuki, MD,[2] Ken Higashimoto, MD,[2] Hidenobu Soejima, MD,[2] and Keiichi Isoyama, MD[1]

Beckwith–Wiedemann syndrome (BWS) is characterized by an accumulation of multiple congenital anomalies. Although patients with BWS are known to have a higher incidence of embryonal tumors, there has been no reports associated with acute leukemia. This report describes the case of a patient with BWS who developed Acute Megakaryocytic Leukemia (AMKL,FAB;M7). Because most patients with BWS present gigantism, the therapy-related toxicity of chemotherapy can be a very serious problem. This patient exhibited no therapy-related toxicity after chemotherapy, suggesting that acute leukemia with BWS may not require a reduction in dosage. Pediatr Blood Cancer. 2010;55:733–735. © 2010 Wiley-Liss, Inc.

Key words: AMKL; Beckwith–Wiedemann Syndrome; chemotherapy; gigantism

INTRODUCTION

Beckwith–Wiedemann syndrome (BWS) is characterized by an accumulation of multiple congenital anomalies. Exophalos, macroglossia, and giantism are considered the most common manifestations [1,2]. Patients with BWS also have a higher incidence of embryonal tumors, such as Wilms tumors [1,2]. This report presents the case of a patient with BWS who developed Acute Megakaryocytic Leukemia (AMKL,FAB:M7).

CASE REPORT

An 1-year- and-3-month-old male presented with a continuous fever. His white blood cell (WBC) count was elevated. The patient was born at 39 weeks of gestation by cesarean section because of fetal distress. At birth, the patient weighed 3,762 g (97th percentile) and had a height of 52 cm (90th percentile). The patient had an omphalocele, macroglossia, bilateral linear creases on the ear lobes, hypoglycemia, and patent ductus arteriosus. As a result, the patient was diagnosed with BWS.

Upon admission the patient's weight and height were 13.2 kg (97th percentile) and 85.8 cm (97th percentile), respectively. The patient displayed cervical lymphadenopathy and hepatosplenomegaly, but kidneys were normal upon an abdominal ultrasound. The patient's WBC count was $119.0 \times 10^3/\mu l$, with 82% leukemic blasts. The hemoglobin level was 6.0 g/dl and the platelet count was $2.2 \times 10^4/\mu l$. Serum levels of lactate dehydrogenase (LDH) were elevated (3,587 IU/L; normal range, 240–530 IU/L). Bone marrow aspiration revealed a normocellular marrow with 85% leukemic blasts (Fig. 1). The leukemic blasts were negative for myeloperoxidase, naphthol AS-D chloroacetate and α-naphthyl butyrate. Surface marker analysis using CD45 blast gating showed the leukemic blasts to be positive for CD4, CD33, CD36, CD41, CD42b, and CD61, thus indicating megakaryocytic origin. Chromosomal analysis showed monosomy 7 with 45, XY, -7, der(10)t(7;10)(q11;p11)ins(10;?)(p11;?). The patient was diagnosed with AMKL. In the patient's leukemic and somatic cells, no methylation of the imprinted domain was observed at the 11p15.5 region (namely, no methylation of DMR-LIT1 and H19-DMR). In addition, no paternal uniparental disomy (patUPD) or CDKNIC mutations were seen. The patient was treated with a low dose cytosine arabinoside (AraC) for cytoreduction. Because there is no report of BWS associated with acute leukemia and because the patient was large for his age, the possibility for therapy-related toxicity due to full dosage induction therapy was a concern. Therefore, the patient received induction therapy according to protocol AML99-Down (AraC 100 mg/m^2 for 7 days; pirarubicin 25 mg/m^2 on days 1 and 2; etoposide 150 mg/m^2 on days 3, 4, and 5), which were lower doses than for conventional induction therapy for patients without Down syndrome. However, the patient failed to achieve remission. He thereafter received full dose chemotherapy according to protocol AML99-inductionC (AraC 500 mg/m^2 on days 1–3 and on days 8–10; idarubicin 8 mg/m^2 on days 1–3; etoposide 200 mg/m^2 on days 8–10). The patient did not experience any therapy-related toxicity thereafter and a complete remission was achieved. He received allogeneic cord blood transplantation (CBT) upon the first remission after three courses of consolidation therapies.

The preparative regimen included busulfan (4.8 mg/kg intravenously × 4 days), etoposide (60 mg/kg intravenously × 1 day), cyclophosphamide (60 mg/kg intravenously × 2 days). The donor was an unrelated female CB (8.0×10^7 nucleated cell/kg) mismatched at 1 HLA loci (HLA-B antigen mismatch). The patient received 0.03 mg/kg tacrolimus and short-term methotrexate (15 mg/m^2 on day1, 10 mg/m^2 on days 3, 6, and 11) for prophylaxis of acute graft-versus-host disease (aGVHD). On day 21, Grade III aGVHD (skin, stage 3; liver, stage 0; gut, stage 3) developed. Therefore, the patient received daily intravenous administration of 2 mg/kg prednisolone (PSL). The skin and gut symptoms resolved

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Conflict of Interest: The authors declare no conflict of interest.

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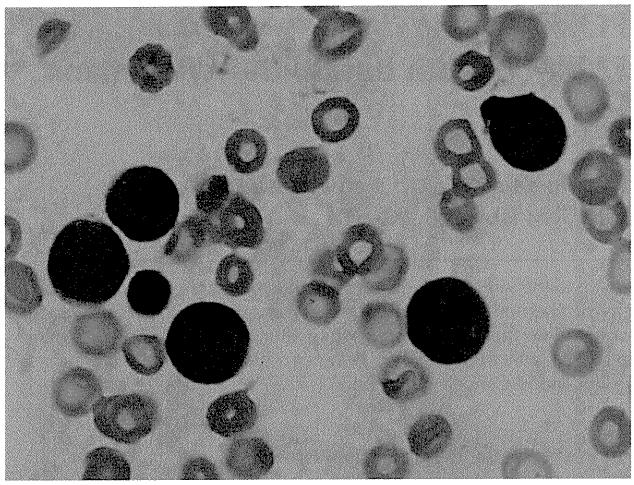

Fig. 1. A bone marrow smear showed small size blasts with high a nucleus-cytoplasm ratio, round nuclei with fine chromatin formation and a few nucleoli. The findings are negative for peroxidase and esterase staining. (Wright-Giemsa staining × 1,000).

after the administration of PSL. The white blood cell count exceeded 1,000/μl on day 36 after CBT. A bone marrow sample obtained on day 45 post-transplant showed 50% leukemic blasts. A chromosomal study showed the same results as the initial diagnosis. FISH analysis revealed 25% donor cells. Tacrolimus treatment was discontinued. However, the skin lesions for aGVHD progressed and intravenous administration of tacrolimus and PSL were restarted. The skin results were stable at stages 2–3 until day 90 and the WBC count was maintained at 2,000–3,000/μl. Thereafter, the skin results gradually resolved, and the WBC count increased. The patient was treated with low dose AraC. However, the patient's general condition did not improve and he died 10 months after CBT from progression disease.

DISCUSSION

Beckwith–Wiedemann syndrome (BWS) is associated with multiple congenital anomalies, exophalos, macroglossia and giantism [1]. Other symptoms include neonatal hypoglycemia, ear creases, hemihypertrophy and cardiac defects [2]. BWS also has a higher incidence of embryonal tumors, such as Wilms tumors [3]. Most cases are sporadic.

BWS is caused by dysregulation of imprinted growth regulatory genes within the 11p15.5 region [4]. The 11p15.5 region contains two independent imprinted domains, IGF2/H19 and KIP2/LIT1. Imprinted genes within each domain are regulated by the imprinting control region (ICR), which, in this case, is either H19-DMR or DMR-LIT1 [5,6]. BWS have been identified DMR-LIT1 loss of DNA methylation, H19-DMR DNA hypermethylation, paternal uniparental disomy (patUPD), and CDKN1C mutations [5,6]. The cause is unknown in approximately 30% of all Japanese BWS patients [6]. In the patient's somatic cells, there was no methylation of DMR-LIT1 and H19-DMR, and there were no patUPD and CDKNIC mutations. As a result, the etiology of BWS remained unclear. Further investigations will therefore be necessary to understand whether the different frequencies of epigenetic and genetic alterations and due to DNA polymorphisms.

BWS predisposes patients to develop embryonal tumors, especially Wilms tumors [2], but there have been no reports of a patient with BWS and acute leukemia. Wilms tumor development in BWS has a strong association with H19-DMR hypermethylation, DMR-LIT1 loss of methylation, and patUPD [7,8]. However, in the patient's leukemic cells, no methylation of DMR-LIT1 or H19-DMR was observed, and no patUPD or CDKNIC mutations were seen either. We did not perform Comparative Genomic Hybridization (CGH) because the patient did not exhibit any methylation of DMR-LIT1 and H19-DMR. To analyze patUPD, we used DNA polymorphic markers for 11p15.5, including tetranucleotide repeats in the tyrosine hydroxylase (TH) and D11S1997, trinucleotide repeats in the D11S2326, dinucleotide repeats in the D11S1318. These were all both paternal and maternal alleles. Therefore, there were no defects at 11p15.5.

The leukemic cells had monosomy 7 with der(10)t(7;10)(q11;p11). This is an atypical association with AMKL. This unbalanced translocation may have contributed to the development of AMKL.

There have so far been no reports of BWS patients who develop acute leukemia. In addition, there is no data indicating optimal therapy that is optimal or tolerable.

The weight and height of the current patient corresponded to the normal values for a 3-year-old and 2-year-old child, respectively. The patient was over 20% of the normal height for his age because of the obesity associated with BWS, and this overgrowth may be linked to increased levels of growth hormones [9], the BWS-associated overgrowth was thought to be balanced. We did not check the patient's insulin-like growth factor levels, which may have been elevated [9]. The patient did not experience any therapy-related toxicity after AML99-inductionC and consolidation therapies. Therefore, no reduction in the dosage of chemotherapy for acute leukemia with BWS was deemed necessary.

The treatment results for children without Down syndrome who have AMKL have been poor [10,11]. In particular, the outcome is extremely poor in children who fail to attain remission or who experience a disease relapse [12]. The current patient underwent allogeneic stem cell transplantation (allo SCT) during the first CR because monosomy 7 was detected.

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Original Article

Organotypic culture of human bone marrow adipose tissue

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The precise role of bone marrow adipose tissue (BMAT) in the marrow remains unknown. The purpose of the present study was therefore to describe a novel method for studying BMAT using 3-D collagen gel culture of BMAT fragments, immunohistochemistry, ELISA and real-time reverse transcription–polymerase chain reaction. Mature adipocytes and CD45+ leukocytes were retained for >3 weeks. Bone marrow stromal cells (BMSC) including a small number of lipid-laden preadipocytes and CD44+/CD105+ mesenchymal stem cell (MSC)-like cells, developed from BMAT. Dexamethasone (10 μmol/L), but not insulin (20 mU/mL), significantly increased the number of preadipocytes. Dexamethasone and insulin also promoted leptin production and gene expression in BMAT. Adiponectin production by BMAT was <0.8 ng/mL under all culture conditions. Dexamethasone promoted adiponectin gene expression, while insulin inhibited it. This finding suggests that dexamethasone, but not insulin, may serve as a powerful adipogenic factor for BMAT, in which adiponectin protein secretion is normally very low, and that BMAT may exhibit a different phenotype from that of the visceral and subcutaneous adipose tissues. BMAT–osteoblast interactions were also examined, and it was found that osteoblasts inhibited the development of BMSC and reduced leptin production, while BMAT inhibited the growth and differentiation of osteoblasts. The present novel method proved to be useful for the study of BMAT biology.

Key words: adipocytes, adipokine, bone marrow adipose tissue, bone marrow stromal cells, dexamethasone, hematopoietic cells, mesenchymal stem cells, organotypic culture, osteoblasts

Bone marrow adipose tissue (BMAT) consists of multiple cell types including mature adipocytes and hematopoietic cells. BMAT has been suggested to function in many aspects of marrow homeostasis such as (i) simply occupying excess space in the marrow cavity; (ii) systemic lipid metabolism; (iii) serving as a localized energy reservoir; and (iv) the regulation of hematopoiesis, osteogenesis and osteoclastogenesis.[1] The precise roles of BMAT in bone marrow homeostasis, however, remain unknown. To investigate the function of the multiple cell types containing BMAT, an appropriate culture system of BMAT seems to be required, but the method has not been established. One of the major reasons for the lack of an appropriate culture system is the difficulty in culturing BMAT, which contains a large number of lipid droplet-embracing mature adipocytes that do not attach to the surface of the culture dish and that are buoyant in the culture medium.

To investigate the roles of BMAT in bone marrow homeostasis, we developed a novel culture system using BMAT fragments embedded in a 3-D collagen gel that was able to easily trap buoyant BMAT. This novel method was based on our previously reported culture method of subcutaneous adipose tissue.[2,3] BMAT *in vivo* is adjacent to bone trabecular surface-lining osteoblasts, suggesting their critical interaction. Thus, we also demonstrated the interaction between BMAT and osteoblasts *in vitro*, as an application of this novel method.

MATERIALS AND METHODS

Culture system

All experimental procedures outlined in the present study were pre-approved by the ethics committee of Saga University and were conducted in accordance with the ethics guidelines of this university. BMAT was obtained from the femoral bone marrow of four female patients (age range,

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60–71 years; mean age, 65.5 ± 5.8 years) who had osteoarthritis of the hip and underwent orthopaedic surgery for arthroplasty at Saga University. Fragmented bone-containing materials (3–10 mL) were placed in Petri dishes containing 10 mL PBS for 10 min. Once the bone fragments had precipitated to the bottom of the dish, the BMAT fragments that remained floating in the PBS were collected. These fragments were then minced within approximately 0.5 mm diameter. The fragments contained numerous mature adipocytes and a small number of mononuclear blood cells that had leukocyte morphology. The mononuclear blood cells were not megakaryocytes or normoblasts, because the starting materials were obtained from the fatty bone marrow. A total of 0.1 mL BMAT fragments was then embedded in 1.0 mL type I collagen gel solution (Nitta Gelatin, Osaka, Japan), and cultured in Ham's F-12 medium supplemented with 10% newborn calf serum and 50 μg/mL gentamicin (Fig. 1a). The medium was exchanged for fresh medium every 2 days. In some cases, 10 μmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA)[4] and 20 mU/mL insulin (Sigma-Aldrich)[5] were also added to the medium upon each exchange.

Histology and morphometry

For histology and morphometry, we fixed the cellular layer gel with 5% formalin and routinely processed the gel to paraffin. We then deparaffinized the sections and stained the cells with HE. The sections were then observed on light microscopy. To detect both the mature adipocytes and preadipocytes, we carried out oil red O staining on samples fixed with osmic acid as previously described.[6,7] We called the spindle-shaped cell types that were found to develop from the BMAT fragments bone marrow stromal cells (BMSC). In the BMSC population, the cells that were S-100 protein positive and demonstrated fine lipid droplets were designated preadipocytes. We then counted the total number of BMSC using a ×20 objective in five randomly chosen areas surrounding the BMAT fragments identified in sections stained for either histochemistry or immunohistochemistry. The percentage of preadipocytes was calculated using the formula (no. preadipocytes)/(total no. BMSC) ×100 (%). Because hematopoietic cells within BMAT demonstrated mononuclear leukocyte morphology in culture, we also examined the expression of the leukocyte markers as described in the following section. Finally, we estimated the formation of bone, cartilage and muscle tissues, which may be organized by BMSC, on the basis of their specific morphology,[8] using HE staining sections of the culture assembly.

Immunohistochemistry and immunofluorescence

We used the rabbit polyclonal S-100 protein antibody (DakoCytomation, Glostrup, Denmark) to detect mature adipocytes and preadipocytes. To identify leukocytes, we used mouse monoclonal antibodies directed against the leukocyte common antigen CD45 (Nichirei, Tokyo, Japan), neutrophil elastase, CD20, CD79a, CD3,[9] PG-M1 (DakoCytomation), and CD163 (NovoCastra Laboratories, Newcastle upon Tyne, UK). Deparaffinized sections were immunostained using the avidin–biotin complex immunoperoxidase (ABC) method as described previously.[10] It has been shown previously that adipose tissue generally contains CD44+/CD105+ mesenchymal stem cells (MSC).[11,12] To detect MSC-like cells, we used mouse monoclonal CD44 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CD105 antibodies (NovoCastra Laboratories). To confirm the co-localization of CD105 and CD44, we carried out double color immunofluorescence on deparaffinized sections using fluorescein isothiocyanate- or rhodamine-conjugated avidin (Molecular Probes, Eugene, OR, USA) as described previously.[2,13] We also carried out double immunohistochemistry using bromodeoxyuridine (BrdU) and each of the antibodies listed here as described previously.[10]

Cell proliferation

We examined cell growth in the culture assembly at 1 week using BrdU (Cell Proliferation Kit, Amersham, Arlington Heights, IL, USA) immunohistochemistry. Briefly, the cells were incubated with 3 μg/mL BrdU after 48 h in culture.[14] A total of 100 cells within BMAT in randomly chosen high-power fields (x20 objective) were counted and the percentage of BrdU intake in both the S-100+ mature adipocytes containing large lipid droplets and the CD45+ leukocytes was calculated. The percentage of BrdU-positive preadipocytes and non-lipid containing BMSC in 100 BMSC was also calculated using this method.

Real-time reverse transcription–polymerase chain reaction

We investigated the expression of peroxisome proliferator-activated receptor (PPAR)γ, adiponectin and leptin using real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from BMAT after 1 week in culture using Isogen (Nippon Gene, Tokyo, Japan). To increase the purity of the complementary DNA (cDNA), total RNA was re-extracted using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). PCR was then performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR was undertaken in a total volume of 20 μL containing Power SYBR green PCR Master Mix (Applied Biosystems) and Quantitect Primers for PPARγ (Hs_PPAG_1_SG), leptin

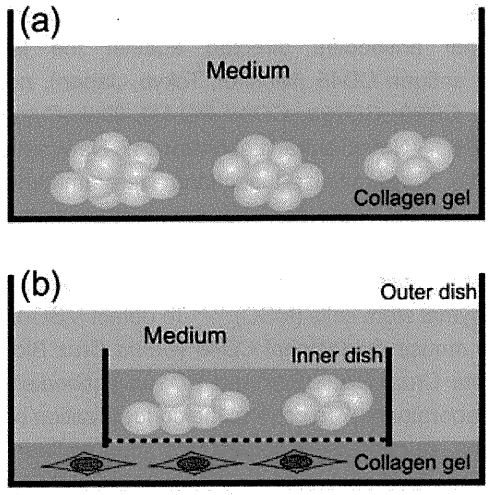

Figure 1 Bone marrow adipose tissue (BMAT) organotypic culture and co-culture systems of BMAT fragments and osteoblasts. (**a**) A total of 0.1 mL of BMAT fragments obtained from femoral bone marrow is embedded in 1.0 mL type I collagen gel solution. (**b**) BMAT fragments and osteoblasts are embedded in collagen gel in inner and outer dishes, respectively. These two layers are completely separated by nitrocellulose membrane.

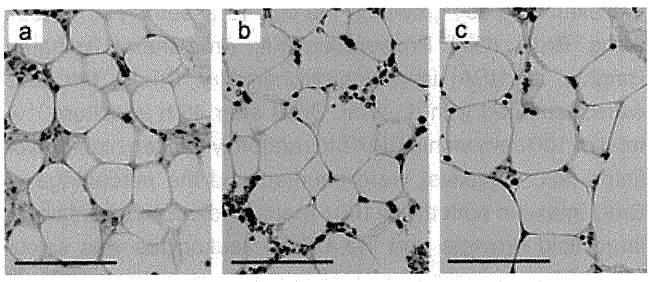

Figure 2 Histology of the human bone marrow adipose tissue (BMAT)-organotypic cultures. (**a**) BMAT immediately after being embedded in the collagen gel (day 0) exhibits mature adipocytes, mononuclear blood cells and erythrocytes, but not spindle-shaped cells. Note that the mature adipocytes contain a large single lipid droplet. At (**b**) 1 week and (**c**) 3 weeks in culture, mature adipocytes and mononuclear blood cells are maintained within BMAT. HE staining. Bars, 100 μm.

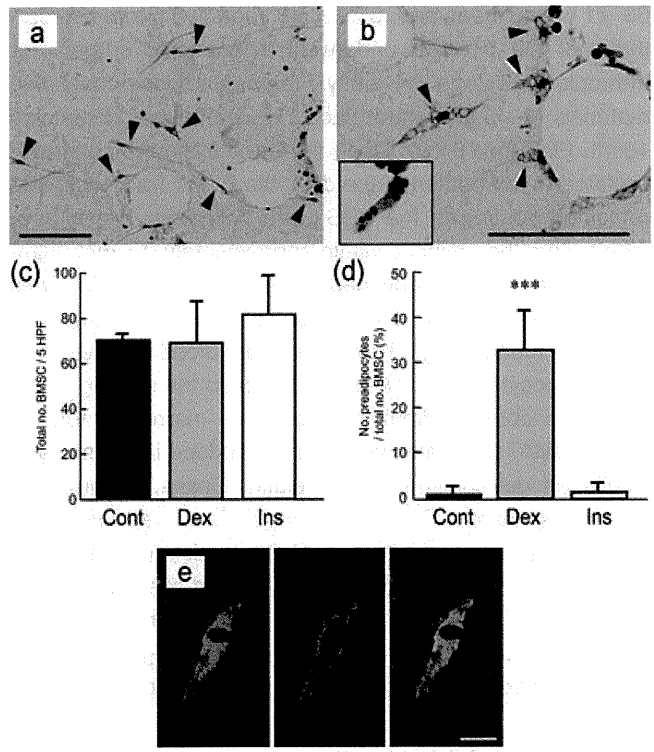

Figure 3 Development of bone marrow stromal cells (BMSC) around bone marrow adipose tissue (BMAT) after 1 week in culture. (**a**) Non-lipid containing BMSC (arrowheads) develop actively around BMAT. (**b**) Addition of 10 μmol/L of adipogenic agent dexamethasone extensively increases the number of preadipocytes (arrowheads) containing oil red O-positive lipid droplets (inset). Bars, 100 μm. (**c**) Dexamethasone and insulin (20 mU/mL) do not affect the total number of BMSC that develop from the BMAT fragments. (**d**) Dexamethasone significantly increases the percentage of preadipocytes among BMSC (***$P < 0.001$ vs control or insulin), while insulin does not. (**e**) Immunofluorescence shows that some BMSC co-express CD105 (green) and CD44 (red). CD105 and CD44 are clearly merged (right panel; bar, 20 μm). The CD105+/CD44+ mesenchymal stem cell-like cells are detected at a rate of 2.8 ± 1.3% in BMSC. Cont, control; Dex, dexamethasone; Ins, insulin. HPF, high-power field.

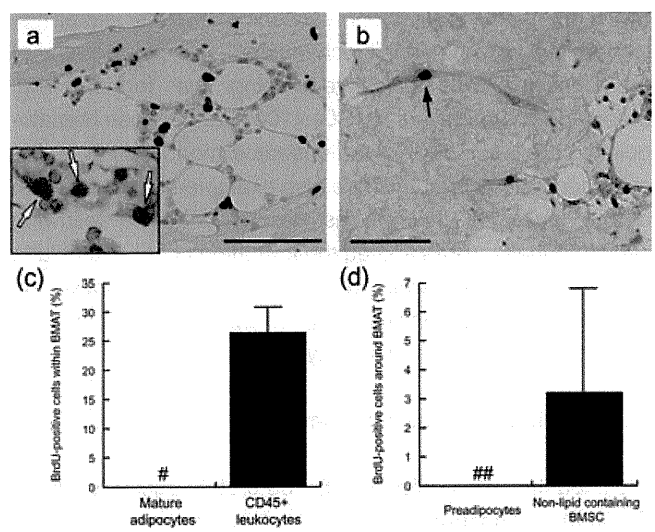

Figure 4 Bromodeoxyuridine (BrdU) immunohistochemistry of (**a**) bone marrow adipose tissue (BMAT) and (**b**) bone marrow stromal cells (BMSC), and BrdU uptake rates of (**c**) mature adipocytes and CD45+ leukocytes, and (**d**) preadipocytes and non-lipid-containing BMSC after 1 week in culture. (**a**) BrdU uptake (black) is detected only in mononuclear blood cells within BMAT, and is not detected in the mature adipocytes. Inset (**a**), the CD45+ (red) leukocytes (arrows) contain BrdU (black). (**b**) BrdU uptake is detected in BMSC (arrow). Bars, 100 μm. (**c**) CD45+ leukocytes exhibit BrdU uptake (26.4 ± 4.7%), whereas mature adipocytes (#) do not. (**d**) Non-lipid-containing BMSC exhibit BrdU intake (3.2 ± 3.7%), while preadipocytes (##) do not.

(Hs_LEP_1_SG), adiponectin (Hs_ADIPOQ_1_SG) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs_GAPDH_2_SG; Qiagen). The expression of each gene was normalized to that of GAPDH.

Adipokine production

We measured adiponectin and leptin levels in the supernatant after 1 week in culture using the human ELISA kits of adiponectin (assay sensitivity: 50 pg/mL; AdipoGen, Seoul, South Korea) and leptin (assay sensitivity: 7.8 pg/mL; R&D Systems, Minneapolis, MN, USA). The adiponectin kit detected its total form.

Effects of soluble factors on adipokine production and gene expression

We next examined the effects of the following factors on adipokine production and gene expression: (i) adipogenic factors of dexamethasone and insulin; and (ii) inflammation-related agents of tumor necrosis factor-α (TNF-α; R&D Systems) and lipopolysaccharide (LPS; *Escherichia coli* 0127 B8, Sigma-Aldrich). One week cultures were stimulated with 10 μmol/L dexamethasone,[4] 20 mU/mL insulin,[5] 2 nmol/L TNF-α[15] or 10 μg/mL LPS[16] for 48 h and analyzed using ELISA and real-time RT-PCR as described in the previous sections.

BMAT-osteoblast interactions

We established co-cultures of the BMAT fragments and osteoblasts (Fig. 1b) as follows. Type I collagen gel (1 mL; Nitta Gelatin) containing 1×10^6 MC3T3-E1 osteoblasts (ATCC, Manassas, VA, USA)[17] was added to a six-well plate (outer dish), and the gel (1 mL) containing 0.1 mL BMAT fragments was poured into a 30 mm diameter dish (inner dish), the bottom of which contained nitrocellulose membrane (Millicell-CM, Millipore, Bedford, MA, USA). The inner dish was then placed on the outer dish and medium added to both dishes. In this system, cells were fed sufficient culture medium in both the inner and outer dishes due to the permeability of the nitrocellulose membrane. The BMAT fragments or osteoblasts cultured alone in the gel served as the controls. Cells cultured in this manner were analyzed using the methods described in the previous section. We also examined the gene expression levels of alkaline phosphatase (ALP), type I collagen, and osteocalcin in the osteoblasts. For gene expression analysis, real-time RT-PCR was undertaken in a total volume of 20 μL containing Power SYBR green PCR Master Mix (Applied Biosystems) and the Quantitect Primer pairs for ALP (Mm_Akp2-1_SG), type I collagen (Mm_Col1a1-1_SG), osteocalcin (Mm_Bglap1-1_SG) or β-actin (Mm_Actb_2_SG) (Qiagen). The expression of each gene was normalized to that of β-actin.

Statistical analysis

Statistical differences between the data obtained in 4–10 independent experiments were analyzed using Student's *t*-test. Values are presented as mean ± SD. $P < 0.05$ was considered significant.

RESULTS

BMAT-organotypic culture

Immediately after being embedded in the gel, the BMAT fragments exhibited numerous mature adipocytes and a small number of mononuclear blood cells and erythrocytes (Fig. 2a). Viable mature adipocytes and mononuclear blood cells were maintained for at least 3 weeks in culture (Fig. 2b,c). The central portions of the fragments demonstrated no significant changes. At 1 week in culture, mononuclear blood cells consisted of B and T lymphocytes, and macrophages (approx. 23.4%, 33.3%, and 36.2%, respectively). In contrast, the peripheral zones of the fragments underwent numerous prominent changes. After 1 week in culture, BMSC (70.0 ± 3.6 cells) developed at the peripheral zones (Fig. 3a,c). In these BMSC the proportion of lipid-containing preadipocytes was 0.75 ± 0.96%. The number of BMSC increased with culture term (3 weeks in culture: 86.0 ± 10.4, $P < 0.05$, vs 1 week; 4 weeks in culture: 93.5 ± 9.5, $P < 0.005$, vs 1 week). The adipogenic factors dexamethasone and insulin had no significant effects on the morphology of mature adipocytes. In addition, these agents failed to affect the total number of BMSC (dexamethasone: 68.8 ± 18.0 cells, $P = 0.89$, vs control; and insulin: 81.3 ± 17.8 cells, $P = 0.26$, vs control; Fig. 3c). But dexamethasone significantly increased the number of preadipocytes (32.0 ± 7.5%, $P < 0.001$, vs control; Fig. 3b,d), a result that was not observed following treatment with insulin (Fig. 3d). Among the BMSC, a few CD44+/CD105+ MSC-like cells (2.8 ± 1.3%) were also observed (Fig. 3e). We did not detect any bone, cartilage or muscle tissues in the culture assembly, based on their specific morphology.[8]

Cell proliferation

Within the BMAT fragments, mature adipocytes demonstrated no BrdU uptake (Fig. 4a,c), while the CD45+

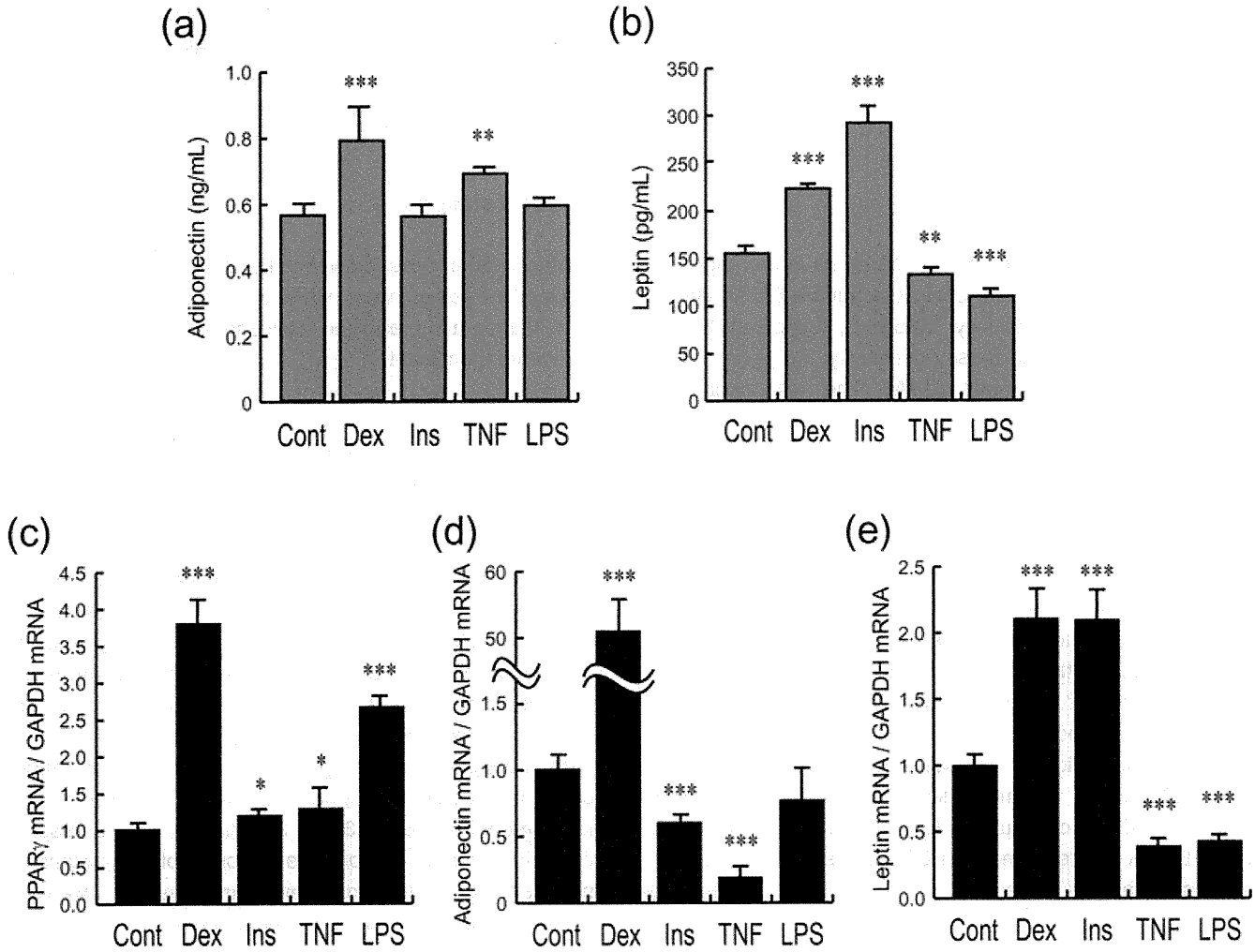

Figure 5 Production of (**a**) adiponectin protein and (**b**) leptin protein, and the mRNA expression of (**c**) peroxisome proliferator-activated receptor (PPAR)γ, (**d**) adiponectin and (**e**) leptin in bone marrow adipose tissue (BMAT) after 1 week in culture, with or without stimulation with several factors for 48 h. The results were analyzed on ELISA and real-time reverse transcription–polymerase chain reaction. (**a**) Adiponectin production in BMAT under all conditions is very low (<0.8 ng/mL). Dexamethasone and tumor necrosis factor-α (TNF-α) increase adiponectin production, while insulin and lipopolysaccharide (LPS) do not. (**b**) Leptin production is detected in cultures without factor stimulation (control). Its production is enhanced with dexamethasone and insulin and inhibited with TNF-α and LPS. (**c–e**) In control cells, mRNA expression of PPARγ, adiponectin and leptin is detected. Dexamethasone significantly enhanced the expression of all the genes. Insulin also promoted expression of PPARγ and leptin, but not expression of adiponectin. TNF-α slightly promoted expression of PPARγ, but clearly inhibited expression of leptin and adiponectin. LPS enhanced expression of PPARγ, but inhibited expression of leptin; it did not affect adiponectin expression. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control. Cont, control; Dex, dexamethasone; Ins, insulin.

leukocytes did (Fig. 4a,c). Around the fragments, non-lipid containing BMSC also showed BrdU uptake (Fig. 4b,d), but the preadipocytes did not (Fig. 4d).

Adipokine production

Treatment with dexamethasone and TNF-α increased adiponectin production, while insulin and LPS treatment did not. Adiponectin production in BMAT under all culture conditions, however, was <0.8 ng/mL (Fig. 5a). We also found that dexamethasone and insulin increased leptin production, while TNF-α and LPS inhibited it (Fig. 5b).

Adipose tissue-specific gene expression

The addition of dexamethasone to cultures enhanced the expression of PPARγ, adiponectin and leptin. Insulin also increased the expression of PPARγ and leptin, while it inhibited the expression of adiponectin. TNF-α addition promoted the expression of PPARγ, while it inhibited adiponectin and leptin expression. LPS treatment enhanced PPARγ

expression and inhibited leptin expression but had no effect on adiponectin expression (Fig. 5c–e).

BMAT–osteoblast interaction

BMSC were found to actively develop in cultures containing only BMAT fragments (Fig. 6a,c). In contrast, the development of BMSC from the BMAT fragments was inhibited at 1 and 3 weeks when cultured in the presence of osteoblasts (Fig. 6b,c). Adiponectin production in BMAT cultured with or without osteoblasts was not significantly affected and remained very low, while leptin production was inhibited in the BMAT and osteoblast co-cultures (Fig. 6d). BMAT also inhibited BrdU uptake by osteoblasts (Fig. 7a–c) and their expression of the differentiation markers ALP, type I collagen and osteocalcin (Fig. 7d). Interestingly, osteoblasts were found to inhibit BrdU uptake in the mononuclear cells within the BMAT fragments (Fig. 8). Given that these mononuclear cells expressed CD45 (data not shown), these cells were most likely leukocytes.

DISCUSSION

The culture of mature adipocytes has proven to be technically difficult in the past due to their buoyancy in culture medium. To resolve this problem, we have established two systems for culturing isolated mature adipocytes: the ceiling culture[6] and the 3-D collagen gel culture.[18] These methods are useful for studying isolated mature adipocyte behavior, but they do not allow for studying BMAT that contains multiple cell types. In the current study we established a novel culture system for human BMAT fragments embedded in collagen gel. This method maintains mature adipocyte and leukocyte survival for >3 weeks. Furthermore, BMSC, which contain a few preadipocytes and CD44+/CD105+ MSC-like cells, develop around the BMAT fragments. Given that appropriate long-term BMAT culture systems have not been established previously, our method may provide a potentially important approach for studying BMAT biology.

In the present study we found that treatment with dexamethasone drastically increased the number of preadipocytes that developed from BMAT. In contrast, treatment with insulin was not found to affect preadipocyte production. Dexamethasone treatment also enhanced the expression of the adipogenic transcription factor PPARγ to a greater degree than insulin, supporting the findings that BMAT has a lower sensitivity for insulin than subcutaneous adipose tissue.[19,20] Using this same culture system with rat subcutaneous adipose tissue, we have previously shown that insulin significantly increases the number of preadipocytes in the culture.[2] These results suggest that dexamethasone, but not insulin, is a powerful adipogenic modifier of BMAT.

Interestingly, adiponectin production by BMAT cultured under all conditions was very low (<0.8 ng/mL), even though dexamethasone and TNF-α treatment enhanced production. In contrast, insulin and LPS had no effect on adiponectin production. Several studies have demonstrated that dexamethasone and insulin would promote adiponectin production in the subcutaneous and visceral adipose tissues, while TNF-α and LPS would inhibit it.[2,21,22] In our preliminary study we detected adiponectin production at a higher level (11.5 ± 0.2 ng/mL) in cultures of subcutaneous adipose tissues obtained from the same patients whose BMAT materials were used here. These results suggest that BMAT exhibits a different phenotype from that of the visceral and subcutaneous adipose tissues in terms of both adiponectin production and response to dexamethasone and insulin treatment.

Dexamethasone and insulin also promoted leptin production, while TNF-α and LPS inhibited it. The mRNA expression of leptin corresponded well with that of leptin protein under all culture conditions. These responses of BMAT were similar to those of the visceral and subcutaneous adipose tissues.[2,20,21] Leptin has recently been shown to promote osteoblast formation and hematopoiesis, but inhibit adipogenesis.[23,24] These findings suggest that leptin contributes to bone marrow osteogenesis and hematopoiesis.

Dexamethasone also induced high levels of PPARγ expression in BMAT, while insulin only slightly enhanced expression levels. These results are consistent with those reported previously.[19,20] Although TNF-α and LPS reportedly inhibit PPARγ expression in the visceral and subcutaneous adipose tissues,[2] we found that they enhanced it in BMAT, albeit only moderately. Leukocyte-linked cells have also been reported to express PPARγ.[25] In addition, we demonstrated that leukocytes are maintained in our BMAT cultures. Thus, it appears likely that leukocytes within BMAT may be responsible for PPARγ expression induced by TNF-α and LPS.

In the present study we detected the proliferation of BMSC and leukocytes, but not mature adipocytes and preadipocytes. Our previous study showed that mature adipocytes and preadipocytes have the ability to proliferate in the culture system of rat subcutaneous adipose tissue.[2] Although the reasons for this discrepancy are unclear, the following possibilities have been raised: (i) differences between BMAT and subcutaneous adipose tissue; (ii) effects of leukocytes within BMAT; and (iii) species differences between human and rat. To address these important issues, further studies are required.

Hematopoietic cells were reported to require the niche produced by stromal cells for their long-term culture.[26] Dexter culture method is suitable for analyzing the interaction between hematopoietic cells and stromal cells.[27] Our method would provide the microenvironment to analyze the

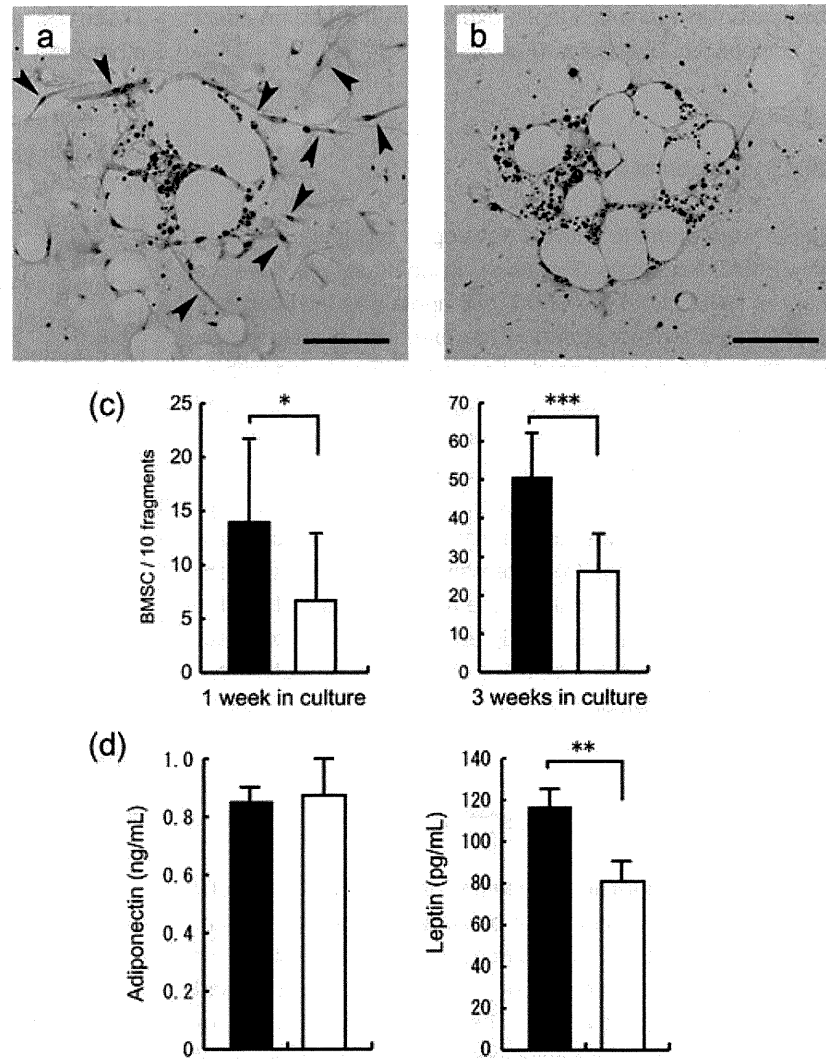

Figure 6 (**a**–**c**) Development of bone marrow stromal cells (BMSC) from bone marrow adipose tissue (BMAT) fragments and (**d**) production of adiponectin and leptin in culture with or without osteoblasts. (**a**) Numerous BMSC (arrowheads) appear around the BMAT fragments in the absence of osteoblasts at 3 weeks. (**b**) In contrast, development of BMSC from the fragments is inhibited in co-culture with osteoblasts. Bars, 100 μm. (**c**) Significant difference is seen between the number of BMSC in culture (□) with or (■) without osteoblasts (*$P < 0.05$ at 1 week; ***$P < 0.001$ at 3 weeks). (**d**) Adiponectin production in BMAT cultured (■) without osteoblasts is very low and (□) with osteoblasts is unaffected. Leptin is detected in cultures (■) without osteoblasts (control), and inhibited (□) with osteoblasts.

interaction between hematopoietic cells and adipocytes as a stromal cell type. To address an interaction between BMAT and hematopoietic cells in more detail, hematopoietic cell-rich marrow obtained from ilium or sternum would be more suitable as materials.

Our co-culture method creates the following active interaction between BMAT and osteoblasts: (i) osteoblasts inhibit the development of BMSC from BMAT, leptin secretion from BMAT and growth of leukocyte-linked hematopoietic cells; and (ii) BMAT in turn inhibits osteoblast growth and differentiation. These interaction-based phenomena took place actively in a paracrine manner, because the osteoblast layer was completely separated from the BMAT layer by the nitrocellulose membrane. Their reciprocal suppressive effects may be involved in the regulatory mechanisms of bone marrow homeostasis. In order to define the mechanisms underlying the osteoblast-BMAT interaction, further studies on the detection of soluble mediators are required.

In conclusion, our culture system established an active interaction between BMAT and osteoblasts. Thus, this method may prove a promising tool for the analysis of interactions between multiple cell types within BMAT, and for the study of skeletal bone marrow pathophysiology during osteoporosis and other hematopoietic disorders.

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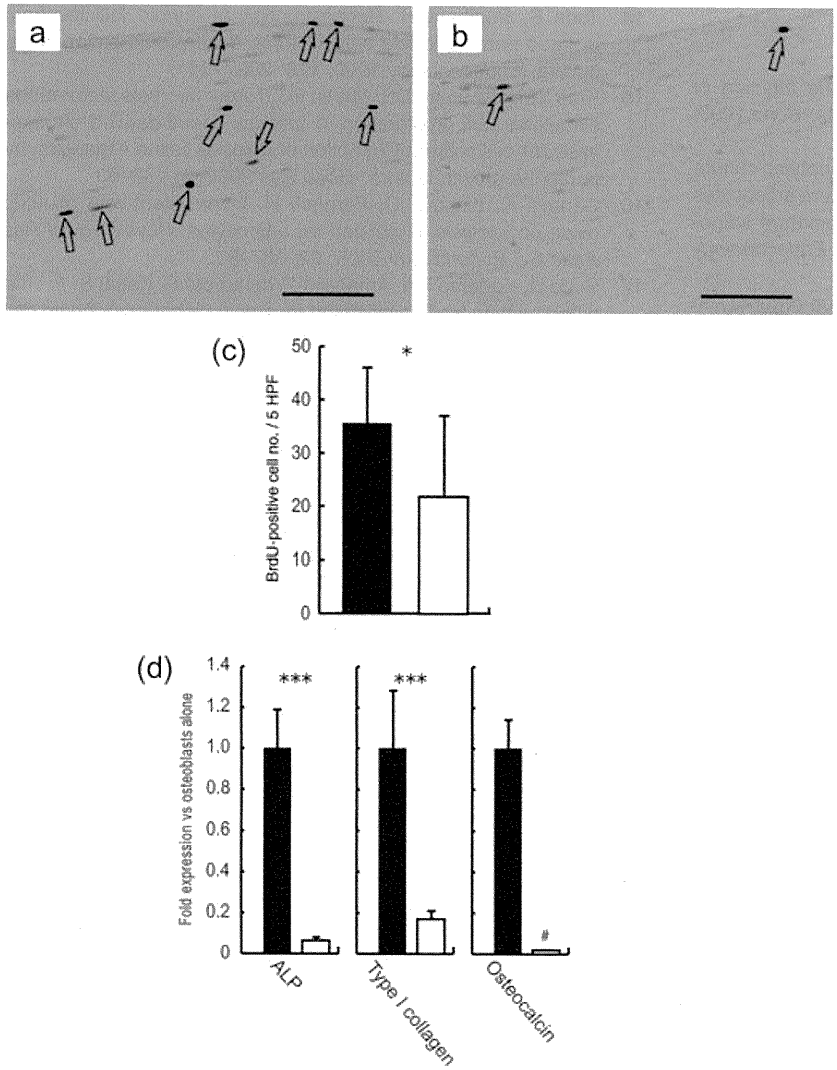

Figure 7 After 1 week in culture, the number of (**a**) bromodeoxyuridine (BrdU)-positive osteoblasts (arrows) without bone marrow adipose tissue (BMAT) fragments is higher than that (arrows) (**b**) with the fragments. Bars, 100 μm. (**c**) Significant difference is seen in the number of BrdU-positive osteoblasts between cultures (□) with and (■) without BMAT fragments (*$P < 0.05$). (**d**) Alkaline phosphatase (ALP), type I collagen and osteocalcin expression in osteoblasts (□) with or (■) without BMAT analyzed using real-time reverse transcription–polymerase chain reaction. BMAT inhibits ALP and type I collagen expression in osteoblasts (***$P < 0.001$). Osteoblasts without BMAT clearly express osteocalcin, whereas the cells with BMAT express it below the detectable range (#). HPF, high-power field.

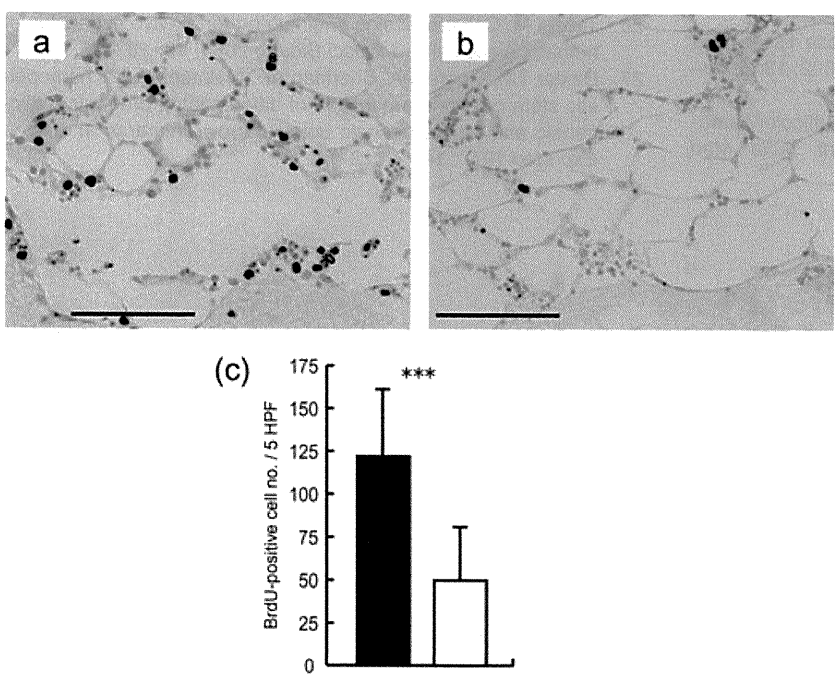

Figure 8 The number of bromodeoxyuridine (BrdU)-positive cells in culture (**a**) without osteoblasts (i.e. bone marrow adipose tissue (BMAT) alone) is higher than that (**b**) in culture with osteoblasts. Bars, 100 μm. (**c**) Significant difference is seen in the number of BrdU-positive cells between cultures (□) with and (■) without osteoblasts (***$P < 0.001$). These findings suggest that osteoblasts inhibit the growth of mononuclear cells within BMAT. HPF, high-power field.

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