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De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor

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ABSTRACT **Autografting of fat pads has a long history in plastic and reconstructive surgery for augmentation of lost soft tissue. However, the results are disappointing because of absorption of the grafts with time. The fate of transplanted fat is linked to adipose precursor cells distributed widely in connective tissues. Adipocyte precursor cells can proliferate and mature into adipocytes even in the adult body depending on microenvironment. When reconstituted basement membrane, Matrigel, supplemented with more than 1 ng/ml bFGF was injected s.c. into 6-week-old mice, the neovascularization induced within 1 week was followed by migration of endogenous adipose precursor cells, and a clearly visible fat pad was formed. The pad grew until 3 weeks after the injection and persisted for at least 10 weeks. Such *de novo* adipogenesis was induced reproducibly by s.c. injection of Matrigel and bFGF over the chest, lateral abdomen, or head. Adipogenesis could be induced even in ear cartilage or in muscle. Thus, our results demonstrated that an abundant population of adipose precursor cells is distributed widely in connective tissues of the adult body and that they migrate into the neovascularized plug of Matrigel for proliferation and maturation. These results suggest a technique of augmenting lost soft tissue in plastic and reconstructive surgery.**

Autografting of fat pads has a long history in plastic and reconstructive surgery for augmentation of lost soft tissue (1). Many uses of autogenous fat grafts, pearls with sizes of a few millimeters in diameter and semiliquid adipose tissue in the treatment of depressed regions or scars in the facial area, have been reported (2). The donor material of semiliquid adipose tissue now is obtained easily by liposuction (3). Despite the enthusiasm for autografting free-fat, however, researchers have been disappointed by progressive absorption of the graft with time (4, 5). When the free-fat autografts were removed and microscopically examined, necrotic adipocytes were observed and replaced by either newly formed host adipocytes or host fibrous tissue in most areas whereas regenerative proliferation of the original fat cells was observed in limited areas. Because the fate of transplanted fat is linked deeply to the proliferation and differentiation of adipose precursor cells, a basic understanding of these cells is important.

It is generally accepted that adipose precursor cells arise from multipotential mesenchymal stem cells, the origin of which remains unknown (6). These stem cells form unipotential adipoblasts. Commitment of adipoblasts leads to the formation of preadipocytes, defined as cells that have expressed early, but not yet late, markers (7) and that have not yet accumulated the intracellular triglyceride droplets. Thus, adipoblasts and preadipocytes are included in interstitial cells having fibroblast-like morphology (8). Although adipoblasts are assumed to appear during embryonic development, it is unclear whether some remain postnatally or whether only preadipocytes are present in the late development. Whatever the stage of the differentiation, such adipose precursor cells are widely distributed in connective tissues and can proliferate and mature into adipocytes even in adult tissues, depending on the microenvironment. For successful autotransplantation of free-fat, these adipose precursor cells need to be transferred together with mature adipocytes and the microenvironment of their regenerative proliferation needs to be maintained at the site of grafting. Alternatively, if one can successfully prepare the microenvironment for the proliferation of endogenous adipose precursor cells, *de novo* formation of fat can be expected even without fat transplantation.

The development of a vascular supply is essential for the generation and maintenance of adipose tissue. In fact, adipocytes and their precursor cells represent only less than one-half of the total cells in adipose tissue; the remaining cells are various blood cells, endothelial cells, and pericytes. Acidic fibroblast growth factor (FGF) (9) and basic FGF (bFGF) (10) are potent inducers of neovascularization. When injected alone s.c. into mice, however, bFGF does not induce neovessel formation, probably because the factor was cleared rapidly from the injection site. On the other hand, Passaniti *et al.* (11) showed that an extract of basement membrane proteins (Matrigel) supplemented with bFGF reconstituted into a gel and supported an intense vascular response when injected s.c. into mice. New vessels and von Willebrand factor antigen staining were apparent in the gel 2–3 days after injection, reaching a maximum after 3–5 days. The hemoglobin content of the gels paralleled the increase of the vessels. This neovascularization could be due to the ability of Matrigel to bind and/or to prevent degradation of FGFs (12) and to stimulate endothelial cell migration. We report here that this neovascularization in reconstituted basement membrane creates an ideal microenvironment for *de novo* adipogenesis by endogenous adipose precursor cells.

When Matrigel supplemented with more than 1 ng/ml bFGF was injected s.c. into nude mice, the neovasculization induced within 1 week was followed by migration of endogenous adipose precursor cells, and a clearly visible fat pad was formed within 2 weeks. The pad grew until 3 weeks after the injection and persisted for at least 10 weeks.

MATERIALS AND METHODS

Preparation of Reconstituted Basement Membrane, Matrigel. Matrigel was prepared from the Engelbreth–Holm–Swarm

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Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; TG, triglyceride.
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sarcoma as described by Kleinman *et al.* (13). In brief, the Engelbreth–Holm–Swarm sarcoma was excised from mice and homogenized in a buffer containing 3.4 M NaCl, 0.05 M Tris·HCl (pH 7.4), 4 mM EDTA, and 2 mM *N*-ethylmaleimide. After centrifugation, the precipitate was extracted overnight at 4°C with an equal volume of 2 M urea solution containing 0.05 M Tris·HCl (pH 7.4) and 0.15 M NaCl and centrifuged at 10,000 × *g* for 30 min. The residue was re-extracted with an equal volume of the urea solution. The two extracts were combined and dialyzed against 0.05 M Tris·HCl (pH 7.4) buffer containing 0.15 M NaCl. The dialysate having a protein concentration of 10–13 mg/ml was adjusted to 10 mg/ml for injection.

Injection of Matrigel Supplemented with bFGF. All animal procedures were conducted under the approval of the Nagoya University Institutional Animal Care and Use Committee. In a preliminary experiment to examine the effect of Matrigel on *in vivo* adipogenesis of 3T3-F442A preadipocytes, the cells cultured to near confluence in DMEM supplemented with 10% fetal calf serum were harvested by scraping with a rubber policeman, suspended in Matrigel with or without 1 μg/ml bFGF (R & D Systems) and immediately injected s.c. into a 6-week-old BALB/c nude mouse over the chest. To follow the time course of *de novo* adipogenesis, 10 6-week-old mice were injected s.c. with 100 μl of Matrigel with or without 100 ng of bFGF over the chest. Each mouse had two injections of Matrigel with and without bFGF supplementation. At 1, 2, 3, 5, or 10 weeks after injections, two mice in each group were killed. The transplants were excised under a microscope, weighed, and analyzed histologically. To study the bFGF dose response, 15 nude mice (6 weeks old) were injected with 100 μl of Matrigel supplemented with the designated amount of bFGF; 0, 0.1, 1, 10, or 100 ng. At 5 weeks after injection, all of the mice were dissected. To study the age dependency, four mice at various postnatal times (1, 2, and 3 weeks) were injected with 100 μl of Matrigel with or without 100 ng of bFGF. Dissection of the mice was performed at 4 weeks after the injections. To compare the injection sites for *de novo* adipogenesis, six mice (6 weeks old) were injected with 100 μl of Matrigel with or without bFGF into s.c. muscular or cartilage regions. At 5 weeks after injections, all samples were excised.

Histology. Specimens were fixed overnight in 10% buffered formalin, dehydrated, and then embedded in paraffin wax. Sections of 5 μm were stained with hematoxylin and eosin and examined. Photographs were taken under a light microscope (Olympus BH-2). For lipid staining, specimens were mounted in OCT compound and rapidly frozen in isopentane CO_2. Then, 10 μm-thick sections were cut with a cryostat at −25°C, air dried, and stained with Sudan IV, which was dissolved in equal volume of acetone and 70% alcohol and later counterstained in Harris hematoxylin.

Triglyceride (TG) Content and Glycerophosphate Dehydrogenase (GPDH) Activity. The specimens were minced and homogenized in 50 mM Tris·HCl buffer (pH 7.4) containing 1 mM EDTA and 1 mM β-mercaptoethanol. The homogenates were disintegrated sonically and centrifuged at 15,000 × *g* for 5 min. The supernatants were used for the assays of TG content and glycerophosphate dehydrogenase activity. For TG assay, a Triglyceride E-test kit from Wako Pure Chemical (Osaka) was used. Glycerophosphate dehydrogenase activity was assayed as described (14). One unit of enzyme activity corresponds to the oxidation of 1 nM of NADH/min.

Statistical Evaluation. Each experiment was repeated independently. Statistical analysis was performed with the aid of the statistical software STATVIEW-J 4.02 for Macintosh.

RESULTS

Neovascularization in Reconstituted Basement Membrane Creates a Microenvironment of *de Novo* Adipogenesis. Because adipocytes that form fat pads *in vivo* are surrounded by well developed basement membranes (15) and the synthesis of basement membrane is enhanced during *in vitro* differentiation of preadipocyte lines (14, 16–18), we first examined the role of basement membrane in adipogenesis by injecting 3T3-F442A preadipocytes s.c. into 6-week-old nude mice over the chest together with reconstituted basement membrane, Matrigel (Fig. 1*B*). Addition of bFGF to the Matrigel plus cells at a dose of 1 μg/ml enhanced the adipogenesis (Fig. 1*D*). To our surprise, a marked adipogenesis also was induced by a control injection of Matrigel in combination with bFGF alone (without 3T3-F442A preadipocytes) (Fig. 1*C*). Injection of bFGF alone (not shown) did not induce any sign of adipogenesis. Except for young mice (see below), injection of Matrigel alone induced poor adipogenesis (Fig. 1*A*). Appreciable adipogenesis was induced only by co-injection of Matrigel with bFGF, suggesting that Matrigel had the ability to bind and/or prevent degradation of bFGF (12) and induced endothelial cells to enter the solidified gel (11). This neovascularization together with the basement membrane environment might create an environment for endogenous adipocyte precursor cells to migrate, proliferate, and differentiate.

***De Novo* Adipogenesis by Injection of Matrigel and bFGF.** Because Matrigel at 4–10°C is liquid, it can be injected s.c. into the mice, where it gels as it is warmed to body temperature. The solidified gels were distinguished easily from surrounding tissues, and a well defined plug between skin and muscles could be excised for the preparation of histological sections. In most experiments, we injected 100 μl of Matrigel. Injection of the larger volume formed wider (not thicker) plugs of solidified gel and created wider fat pads, but there was a limit to the volume of Matrigel that could be retained as blisters just after the injection. Fig. 2 depicts the process of *de novo* adipogenesis when 100 μl of Matrigel was co-injected s.c. with 1 μg/ml

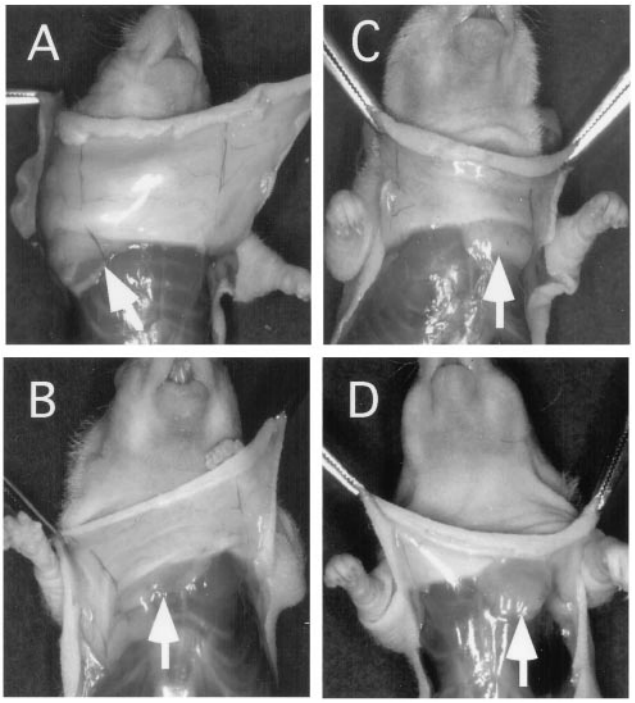

FIG. 1. Enhanced adipogenesis of 3T3-F442A cells and *de novo* adipogenesis by Matrigel in combination with bFGF. 3T3-F442A preadipocytes were suspended in Matrigel with (*D*) or without (*B*) 1 μg/ml bFGF, and 100 μl of the cell suspension containing 2×10^6 cells was s.c. injected into a BALB/c nude mouse over the chest. As the control, 100 μl of Matrigel with (*C*) or without (*A*) 1 μg/ml bFGF also was injected. Arrows indicate fat pads formed 5 weeks after the injection.

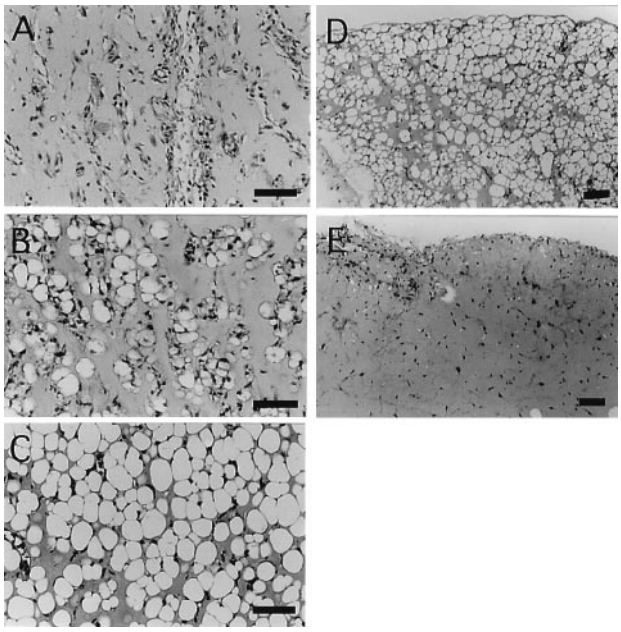

FIG. 2. Process of *de novo* adipogenesis by injection of Matrigel and bFGF. Matrigel was s.c. injected together with 1 μg/ml bFGF over the chests of 6-week-old mice, and well defined plugs between skin and muscle were excised for histological staining with hematoxylin and eosin. Neovascularization in Matrigel is completed within 1 week, and blood vessels with a clear endothelium lining are formed (A). The neovascularization was accompanied by invasion of fibroblast-like cells that differentiate into adipocytes within 2 weeks (*B*). The population and size of the adipocytes increased until 5 weeks after injection (*C*). The fat plugs formed *de novo* by injection of Matrigel and bFGF were preserved for 10 weeks (*D*) whereas the plugs formed by injecting Matrigel alone lacked many cells (*E*). (Bar = 100 μm.)

bFGF into 6-week-old mice. Reproducing the results reported by Passaniti *et al.* (11), the neovascularization in Matrigel was completed within 1 week after the injection, and well developed blood vessels were formed with a clear endothelium lining (Fig. 2*A*). This neovascularization was accompanied by invasion of fibroblast-like cells into Matrigel. Probably because adipose precursor cells were included in these fibroblast-like cells, differentiation of mature adipocytes was observed along with the neovessels 2 weeks after the injection (Fig. 2*B*). Some adipocyte differentiation was observed locally by 1 week after the injection at the periphery of the Matrigel (result not shown). The population and size of the mature adipocytes increased during 3–5 weeks after the injection, and large adipocytes became dominant at the site of injection of Matrigel and bFGF (Fig. 2C). Finally, injected Matrigel appeared to be absorbed, and the space was replaced by mature adipocytes without any sign of inflammatory reaction. The adipose tissue formed *de novo* by injection of Matrigel and bFGF was preserved for at least 10 weeks (Fig. 2*D*). In contrast, the plugs formed by injecting Matrigel alone remained quiescent and had few adipocytes, only in the peripheral area on the skin side (Fig. 2*E*).

Frozen sections of the plugs were evaluated 5 weeks after the injection. When the sections were stained with Sudan IV, only a few adipocytes were stained in the plugs formed by Matrigel alone (Fig. 3*A*) whereas abundant adipocytes were stained in the plugs formed by injection of Matrigel and bFGF (Fig. 3*B*). Adipocyte differentiation in the plugs formed by injecting Matrigel and bFGF was confirmed biochemically by assessing TG content of 0.08 mg and glycerophosphate dehydrogenase activity of 60 unit/mg of the plug.

Dependency of *de Novo* Adipogenesis on bFGF Dose, Animal Age, and Injection Site. Matrigel contains a trace of bFGF, with the amount dependent on the preparations (19). Thus,

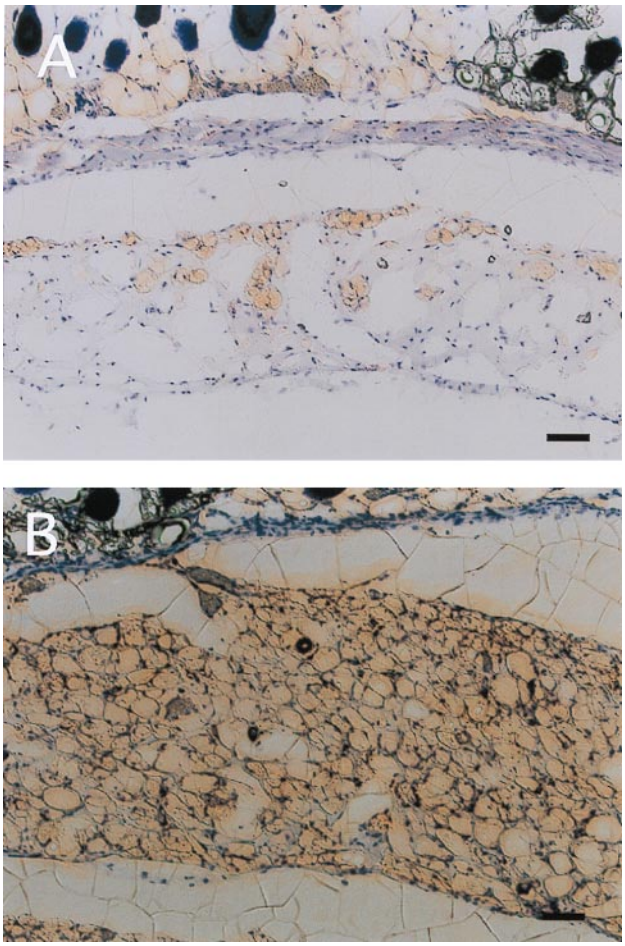

FIG. 3. Lipid staining of plugs formed by Matrigel injection together with or without bFGF. Frozen sections of the plugs 5 weeks after injecting Matrigel together with (*B*) or without (*A*) bFGF were prepared and stained with Sudan IV followed by counterstaining in Harris hematoxylin. Other details as in Fig. 1. (Bar = 100 μm.)

precise estimation of the dependency of adipogenesis on bFGF dose may be difficult. Nevertheless, the weight of plugs formed 5 weeks after injecting 100 μl of Matrigel increased 3-fold by supplementing with bFGF at 1 ng/ml and was maximal at 1 μg/ml with more than a 5-fold increase in weight (Fig. 4*A*). When histological sections of the plugs were compared, 1 ng/ml of bFGF was enough to induce adipogenesis in the Matrigel plug, and the maximal response was observed at 1 μg/ml (Fig. 4*B*).

Injection of Matrigel into young (1- to 3-week-old) mice also caused intense adipogenesis, but the induction did not show clear dependency on supplemented bFGF (result not shown). Limited examples showed *de novo* adipogenesis by injection of Matrigel alone. This may be due to either abundant adipose precursor cells present in young animals that responded to the trace of bFGF in the Matrigel preparation or an active supply of endogenous bFGF to the Matrigel plug from surrounding tissues.

Injections (s.c.) of Matrigel together with bFGF over the chest, over the lateral abdomen, and over the head induced reproducible adipogenesis. The size of inducible fat pads and the homogeneity of adipocytes differed depending on injection sites. A larger volume of Matrigel could be injected over the abdomen to induce larger fat pads. The volume of Matrigel that could be injected s.c. over the head was limited, but histological sections of fat pads formed in the periosteum showed a homogeneous population of adipocytes. Co-injection of Matrigel and 1 μM bFGF into ear cartilage, leg muscle, or

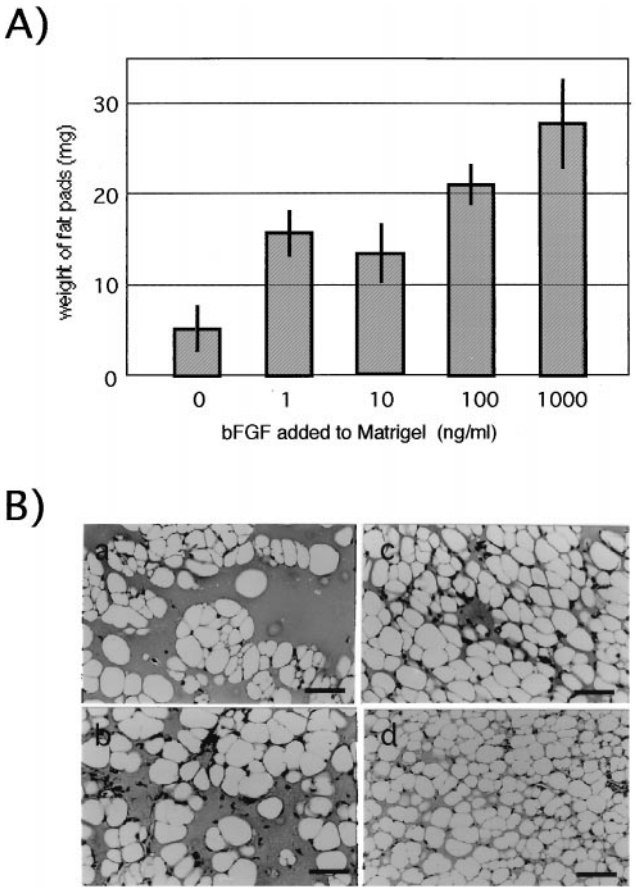

FIG. 4. Dependency of *de novo* adipogenesis on bFGF dose. Six-week-old mice were s.c. injected with Matrigel containing 0, 1, 10, 100, or 1,000 ng/ml bFGF (two injections into three mice) over the chest. (*A*) Averaged weight with SE of the Matrigel plugs excised 5 weeks after the injection. (*B*) Histological staining with hematoxylin and eosin of the tracts formed by injecting Matrigel containing 1 (a), 10 (b), 100 (c), or 1,000 (d) ng/ml bFGF. (Bar = 100 μm.)

masticatory muscle also induced angiogenesis followed by fat pad formation, with a less homogeneous population of adipocytes.

In preliminary experiments of testing other growth factors, platelet-derived growth factor (PDGF) was found to be as potent as bFGF when co-injected with Matrigel. Despite strong adipogenic effects of growth hormone, insulin, and insulin-like growth factor 1 (IGF-1) reported for *in vitro* differentiation of cloned adipose precursor cell lines, these growth factors were less potent in inducing *de novo* adipogenesis.

To explore a substitute for Matrigel, acid-extracted collagen from mouse tail was injected together with bFGF. To get long term and slow supply of bFGF, micro-carrier beads for cell culture were injected s.c. after equilibrating with bFGF-containing PBS. None of these treatments induced *de novo* adipogenesis.

DISCUSSION

The hyperplastic formation of adipose tissue in aged animals by feeding with a high carbohydrate or high fat diet has been studied intensively. The life-long potential of adipose precursor cells to make new adipose tissues has been demonstrated in many experiments on rodents. The fat depots of old mice contain large amounts of early markers of adipocyte differentiation (20). A significant population of stromal–vascular cells from s.c. fat tissues of old men and women has been shown to differentiate *in vitro* into adipocytes (21). These results altogether indicate that adipose precursor cells are distributed widely in adult connective tissues and that their proliferation and differentiation can be enhanced depending on the microenvironment. Here, we showed that such a microenvironment can be created *in situ* by injection of Matrigel. Once neovascularization was enhanced by bFGF, endogenous adipose precursor cells actively migrated into the Matrigel plug and differentiated into adipocytes. Invasion of many fibroblast-like cells into the neovascularized plug of Matrigel suggested the possibility that mesenchymal stem cells with fibroblast morphology remained even in adult body and differentiated into the adipocytes. Such *de novo* adipogenesis could be induced in various s.c. connective tissues and even in muscle and ear cartilage. An important clinical implication of this observation is the cause of fat deposition after involution of capillary hemangiomas (22). Capillary hemangiomas are common tumors that arise largely in s.c. tissues of 3- to 4-week-old children, increase in size for several months up to 1 year, and then start to regress to result in complete resolution by the age of 7 years. Among many other immunohistochemical markers, high expression of bFGF together with vascular endothelial growth factor has been observed in the proliferating and involuting phases (23). If one can assume that endothelial cells produce an unknown adipogenic factor, *de novo* adipogenesis in the neovascularized plug of Matrigel may show the mechanism of fat deposition after involution of capillary hemangiomas. This mechanism implies that injection of endothelial cells together with Matrigel would give faster formation of adipose tissue. For clinical application, however, rapid neovasculization within 1 week of injection of Matrigel and bFGF might be more efficient for the reconstruction of adipose tissue.

In a long history of autografting fat pads for augmentation of lost soft tissue, many improvements of the technique have been tried to prevent absorption of the grafts with time (1). These include local treatment of the implantation bed with insulin and transplanting small grafts. Partial success was achieved by supplementing the grafts with bFGF (24). We show here that transplantation of fat is not necessary, but injection of Matrigel in combination with bFGF is enough to reconstruct adipose tissue at desired loci of the body surface. Histological sections of newly formed fat pads revealed that most of the injected Matrigel was absorbed and replaced by newly differentiated adipocytes. This indicates that Matrigel is not a permanent material but an absorbable material. When injected into nude mice, at least, no sign of intensive inflammation reaction was observed. Our results thus open a new technique of augmentation of lost soft tissues in the plastic and reconstructive surgery. A possible substitute of Matrigel for human therapy might be basement membrane extractable from placenta. For future design of artificial substitute, a responsible molecule among basement membrane components needs to be identified.

Taking advantage of *in vitro* differentiation of cloned adipose precursor cell lines such as 3T3-L1, 3T3-F442A, TA-1, and Ob17, many adipogenic and anti-adipogenic factors have been demonstrated. In this evaluation, FGFs have been classified into anti-adipogenic factors together with PDGFs, epidermal growth factor, and transforming growth factor β. Acidic fibroblast growth factor and bFGF prevented the expression of the late adipocyte-specific genes in mouse TA-1 cells and abolished their expression in a fully differentiated rat adipocyte line (20). FGFs as well as PDGF antagonized the NADPH-dependent H_2O_2 generation and prevented the adipose conversion of 3T3-L1 cells (25). Together with epidermal growth factor and PDGF, FGFs suppressed the late marker of adipocytes in human adipose precursor cells (26). However, *de novo* adipogenesis in Matrigel demonstrated paradoxically that bFGF is the most potent factor of adipogenesis. PDGF was as

potent as bFGF whereas growth hormone, insulin, and IGF-1 were less potent in inducing *de novo* adipogenesis. During the differentiation of 3T3-F442A adipoblasts, growth hormone primes their responsiveness to IGF-1, which in turn causes a mitogenic expansion of preadipocytes (27). Insulin and IGF-1 are known to induce the terminal differentiation of 3T3-L1 preadipocytes (28, 29). Less potent induction of *de novo* adipogenesis by growth hormone, insulin, and IGF-1 is again paradoxical. These results show that apparent enhancement of *de novo* adipogenesis by bFGF and PDGF was due to enhanced neovascularization, which is a prerequisite of *in vivo* adipogenesis, and this environment is established *in vitro* by optimization of the culture conditions. Angiogenic activity of PDGF may not be well established. As far as co-injection with Matrigel, however, our sections of Matrigel plug showed active angiogenesis by PDGF as observed by Passaniti *et al.* (11). Our observation thus showed that the *in vitro* differentiation system does not necessarily reflect *in vivo* adipogenesis.

De novo adipogenesis by co-injection of Matrigel with bFGF suggests that the function of basement membrane is to bind and prevent degradation of bFGF (12). Nevertheless, there remains a possibility that basement membrane has additional functions such as supporting the proliferation and differentiation of adipose precursor cells. In primary culture of adipose tissue stromal–vascular cells, Hausman *et al.* (30) showed that coating culture dishes with Matrigel enhanced attachment and spreading of preadipocytes whereas spreading of nonpreadipocytes was antagonized. Coating the dishes with laminin reproduced this selective spreading of preadipocytes whereas type IV collagen had no influence. This suggests that laminin supported selective proliferation of preadipocytes among the fibroblast-like cells invading the Matrigel plugs. Our effort at identification of responsible components of Matrigel by separating a urea extract of Engelbreth–Holm–Swarm tumor by using a urea–DEAE Sepharose column, on the other hand, showed that the situation is not simple. The column gave fractions enriched with type IV collagen, laminin, and perlecan although they cross-contaminated each other because of strong association even in 5 M urea. None of the fractions induced *de novo* adipogenesis when injected in combination with bFGF. Dilution of the components by mixing fractions made the reconstitution experiment difficult, but our efforts so far suggest that the architecture of basement membrane constructed by laminin, type IV collagen, and perlecan altogether (31) is required for the *de novo* adipogenesis.

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