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De novo adipogenesis for reconstructive surgery

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Abstract

Autografting of lost soft tissue is an important subject of the plastic and reconstructive surgery and autograft of fat pads has been only technique for this goal. However, the results are disappointing because of absorption of the grafts with time. Adipoblasts or adipocyte precursor cells distribute widely in connective tissues and they can proliferate and mature into adipocytes even in the adult body. In experiments using mice, we found that *de novo* adipogenesis of endogenous precursor cells can be induced by injecting reconstituted basement membrane, Matrigel, supplemented with more than 1 ng/ml of bFGF. This adipogenesis was reproducibly induced by subcutaneous injection over the chest, lateral abdomen or head. Adipogenesis was induced even in ear cartilage or in muscle. To evaluate the possibility of future application of this *de novo* adipogenesis to plastic and reconstructive surgery, we have reviewed updated knowledge of the adipogenesis.

Introduction

Autografting of fat pads has a long history in plastic and reconstructive surgery for augmentation of lost soft tissue (Billings and May, 1989). Many uses of autogenous fat grafts, pearls and semiliquid adipose tissue in the treatment of depressed regions or scars in the facial area have been reported (Ellenbogen, 1986). Despite the enthusiasm for improved operation of free fat-autografts, however, researchers have been disappointed by progressive absorption of the graft with time (Teimourian et al., 1989; Ersek, 1991). When the autografts were removed for microscopic examination, necrotic adipocytes were observed and replaced by host fibrous tissue in most areas while regenerative proliferation of the original fat cells were observed in limited areas. The donor material of semiliquid adipose tissue can now be obtained by liposuction (Bircoll and Novack, 1987) but the amount is not enough for augmentation of wide area.

It has been generally accepted that the commitment of adipoblasts, unipotential precursor of adipocytes, completes during the embryonic development. Postnatal supply of adipoblasts has not been expected. This is the reason why the surgery for argumentation of lost fat tissue has been focused only on transplantation of pre-formed fat pads. If one can successfully prepare the microenvironment for commitment of adipoblasts in adult body, on the other hand, the formation of fat pads can be expected without transplantation. Our recent observation of *de novo* adipogenesis in mice at the site of injection of basement membrane suggested that an abundant population of adipose precursor cells is distributed in connective tissues of adult body and that they can migrate, proliferate and maturate to form fat pads with desired size and at desired site. Since this result implies a novel technique of augmenting lost soft tissue, we here reviewed the cellular and molecular mechanism of adipogenesis.

In vivo adipogenesis

Adipocytes belong to the mesenchymal stem-lineage, whose members are *fibroblasts*, cartilage cells (chondrocytes), bone cells (osteoblasts/osteocytes) and smooth muscle cells. The members are not only related but are also interconvertible to each other. This family plays a central role in the support and repair of various connective tissues and their adaptability is important for our body to respond to many types of damage. *Fibroblasts* are the least specified cells in the family and distribute throughout the body where they secrete large amount of extracellular matrix. It is highly possible that this population of *fibroblasts* contains the putative mesenchymal stem cells having the potential of differentiation into various cells of connective tissues. When the tissue is damaged, *fibroblasts* nearby migrate into the wound, proliferate and repair the tissues. In such occasion, it is possible that *fibroblasts* may commit to be adipoblasts.

Adipocytes are believed to arise from putative multipotential mesenchymal stem cells (Johnson and Grenwood, 1988) which create unipotential adipoblasts. From an embryological perspective, the formation of adipoblasts is believed to take place during the embryonic development and such adipoblasts are thought to be the only source of adipocytes during postnatal period. Due to the lack of molecular markers for the adipoblasts, however, nobody has ever confirmed that the commitment of adipoblasts completes during the embryogenesis. Future progress in the molecular biology of adipogenesis might reveal the adipoblast formation in adult body. Commitment of adipoblasts leads to the formation of preadipocytes which have distinct morphology and have been defined in rodent as *interstitial cells* (Bukowiecki et al., 1982; Bukowiecki et al., 1986; Geloen et al., 1988; Geloen et al., 1989a, b), *nonlipid-filled mesenchymal cells* (Miller et al., 1983) or *poorly differentiated* mesenchymal cells (Ochi et al., 1991). Maturation of these preadipocytes initiates by the accumulation of fat droplets, which then coalesce and enlarge until the cells is highly distended with a thin rim of cytoplasm around the mass of lipid.

Whatever the stage of the differentiation, such precursor cells are widely distributed in connective tissues and can proliferate and mature into adipocytes even in adult body depending on the microenvironment. The hyperplastic formation of adipose tissue in aged animals by feeding with a high-carbohydrate or high-fat diet is an evidence for this postnatal differentiation. 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 amount of early markers of adipocyte differentiation (Ailhaud, 1990). A significant population of stromal-vascular cells from subcutaneous fat tissues of old men and women has been shown to differentiate *in vitro* into adipocytes (Hauner et al., 1989). These results altogether indicate that adipose precursor cells are widely distributed in adult connective tissues and their proliferation and differentiation can be enhanced depending on the microenvironment.

As an element of such microenvironment, important role of basement membrane has been recognized. According to Napolitano (Napolitano, 1963), who followed the adipocyte differentiation from fibroblast-like cells of developing fat tissue of newborn rats, the formation of basement membrane-like structure around the cells was the first ultrastructural change seen. The synthesis of basement membrane proteins is enhanced during *in vitro* differentiation of the preadipocyte lines (Kuri-Harcuch et al., 1984; Aratani and Kitagawa, 1988). We have ever shown that ascorbic acid phosphate, a stable and long acting derivative of ascorbic acid, stimulates the synthesis of type IV collagen of 3T3-L1 preadipocytes and accelerates their adipose conversion (Ono et al., 1990). We recently found that the isoform of laminin synthesized by 3T3-L1 preadipocytes is laminin-8 and that the gene expression of component subchains of $\alpha 4$, $\beta 1$ and $\gamma 1$ is enhanced depending on the differentiation (Niimi et al., 1997).

The development of microvascular system is another element of the generation and maintenance of adipose tissue. In fact, adipocytes and their precursor cells represent only less than a half of the total cells in adipose tissue and the remaining cells are various blood cells, endothelial cells and pericytes. Production of angiogenic factors depending on the adipose conversion of cultured cell lines has been demonstrated (Wilkison et al., 1991). This suggests that adipoblasts induce microvascularization in the surrounding connective tissue to get blood supply for adipogenesis. Conversely, well vascularized connective tissue might be the favorable area for adipoblasts to migrate and differentiate. We previously showed that bovine aortic and pulmonary endothelial cells synthesize laminin-8 ($\alpha 4\beta 1\gamma 1$) together with laminin-1 ($\alpha 1\beta 1\gamma 1$) and synthesis of laminin-8 is enhanced under angiogenic conditions (Tokida et al., 1990).

Our finding of laminin-8 production depending on the adipose conversion of 3T3-L1 preadipocytes (Niimi et al., 1997) suggests an important role of laminin-8 in organization of microenvironment for adipogenesis.

In vitro adipogenesis

Taking advantage of *in vitro* adipogenesis of cultured cell lines, considerable information of transcription factors involved in adipogenesis has been accumulated in the last decade. CCAAT-enhancer-binding proteins (C/EBPs), peroxisome proliferator activated receptors (PPARs) and adipocyte determination and differentiation-dependent factor 1 (ADD1) are now established to be three major families of transcription factors cross-talking each other during adipogenesis. The master gene of adipogenesis is not known yet, but many experiments of ectopic expression of these genes *in vitro* have demonstrated that *fibroblastic cells* having poor potential of the adipose-conversion can be adipocytes.

C/EBPα is expressed in several tissues such as liver, lung and intestine in addition to adipose tissue (Birkenmeier et al., 1989). Nevertheless, C/EBPα binds to the promoter region of adipocyte specific genes including 442/adipocyte P2 (aP2), stearoyl CoA desaturase 1 (SCD1), glucose transporter 4 (GLUT4) and phosphoenolpyruvate carboxykinase (PEPCK) (Cheneval et al., 1991). Expression of antisense C/EBPα mRNA in 3T3-L1 cells inhibits the adipose-conversion (Lin and Lane, 1992). Ectopic expression of C/EBPα in 3T3-L1 cells promote the adipose-conversion even in the absence of 1-methyl-3-isobutylxanthine (MIX) and dexthametasone (DEX), the inducers of normal adipose-conversion of 3T3-L1 cells (Lin and Lane, 1994). Furthermore, fibroblasts having low potential of the adipose-conversion can differentiate by ectopic expression of C/EBPα (Freytag, 1994). These results demonstrate that C/EBPα plays an important role in adipogenesis. Since C/EBPα expression is observed at relatively late stage of the adipose-conversion, however, it is unlikely that this transcription factor can function as the master gene.

Two additional C/EBPs, C/EBPβ and δ, are known to be involved in the adipose-conversion. They have well-conserved leucine zipper domain which binds to *cis*-element of the same adipocyte specific genes as C/EBPα. They are also expressed in various tissues other than adipose tissue but their expression prior to C/EBPα during normal adipose conversion of 3T3-L1 cells suggests the function at upstream of C/EBPα (Cao et al., 1991). Ectopic expression of C/EBPβ in fibroblasts can also promote the adipose-conversion by activating PPARγ (see below) (Wu et al., 1995). Ectopic expression of C/EBPδ can promote adipose-conversion of 3T3-L1 cells as well but not of fibroblasts (Wu et al., 1996). C/EBP-liver-enriched inhibitory protein (LIP) and liver-enriched transcription activator (LAP) are additional isoforms of C/EBPs produced by alternative splicing of C/EBPβ pre-mRNA. Since C/EBP-LIP lacks transactivation domain at amino-terminus, it can act as dominant-negative factors of C/EBPs (Descomb et al., 1991). C/EBP homologous protein-10 (CHOP-10)/GADD153 was screened from cDNA library of 3T3-L1 adipocyte based on interaction with leucine zipper domain of C/EBPβ. CHOP-10/GADD153 also lacks a functional DNA-binding domain because alanine and aspargine residue in basic region are alternated to proline and glycine residue, respectively (Ron et al., 1992). The role of these potential dominant negative factors is open to further study.

PPARs are classify into the nuclear hormone receptor family and include three distinct members of PPARα, γ and β (also called δ, NUC-1 or FFAR). They have well conserved DNA-binding domain whereas distinct ligand-binding domains. Identification of PPARγ as an adipogenic transcription factor came from the studies on aP2 gene activation. Spiegelman et al. found a novel differentiation-dependent factor, ARF6, which binds to two *cis*-elements of ARE-6 and -7 (Graves et al., 1992). These *cis*-elements showed homology to DR-1 (direct repeat of hormone response element spaced by one nucleotide), which is known to be the binding site for RXR/PPAR dimer. Alternative splicing of PPARγ pre-mRNA produces isoforms of PPARγ1 and γ2 (Tontonoz et al., 1994a). Whereas PPARγ1 is widely expressed in many tissues, PPARγ2 is expressed specifically in adipose tissue. In addition, their expression is significantly increased at early stage of the adipose-conversion of 3T3-L1 cells. The importance of PPARγ2 in adipogenesis was clearly demonstrated by the adipose-conversion of fibroblasts caused by its ectopic expression (Tontonoz et al., 1994b).

PPARα and δ seem to play physiological roles distinct from PPARγ. The ligand of PPARα has been determined to be leukotriene B4 fibrate (Devchand et al., 1996) whereas that of PPARγ is either 15-deoxy-$\Delta^{12,14}$-PGJ_2 (prostaglandin J2 derivatives) or

antidiabetic thiazolidione, natural and synthetic ligand, respectively (Foman et al., 1995; Kliewer et al., 1995). Their expression is not specific to adipose tissue. Recently, Brun et al. compared three PPAR isoforms in terms of their ability of inducing adipogenesis (Brun et al.,1996). PPARα is able to induce adipogenesis only in the presence of the activators while PPARδ is not. Coexpression of PPARγ with C/EBPα in fibroblast can induce adipogenesis powerfully even in the absence of PPARγ activator, suggesting that they can promote adipogenesis synergistically. However, PPARα and δ did not cooperate with C/EBPα.

The third transcription factor involved in adipogenesis is ADD1, which has a basic helix-loop-helix-leucine zipper (bHLH-LZ) structure. ADD1 can bind to *cis*-element containing E-box (CANNTG) through the basic domain and may form homo- or heterodimer through the HLH domain (Tontonoz et al., 1993). Interestingly, a human homologue of ADD1 was independently found as sterol response element-binding protein-1 (SREBP-1). SREBP-1 is involved in cholesterol metabolism and binds to non-E-box motif of SRE (sterol response element). ADD1 has unusual ability of binding to both E-box motif and non-E-box motif (Kim et al., 1995). The role of ADD1 in adipogenesis was investigated by ectopic expression experiment. Expression of dominant-negative form of ADD1 which can not bind DNA inhibited not only the adipose-conversion of 3T3-L1 cells but also the expression of adipocyte specific genes. Expression of wild type ADD1 in fibroblast could promote the adipose-conversion after the treatment with MIX, DEX, insulin and PPAR activator (ETYA), and induce the expression of fatty acid synthetase and lipoprotein lipase genes. Moreover, ectopic expression of both ADD1 and PPARγ induce the significant increase in transcriptional activity of PPARγ (Kim et al., 1996).

Recent scenario of the adipose-conversion regulated by cross-talk of these transcription factors is as the following. First, expression of C/EBPβ and δ are increased in respond to hormonal signals such as glucocorticoid and insulin, which in tern induces PPARγ expression. ADD1/SREBP-1 controls fatty acid metabolism and cholesterol homeostasis and lead to the activation of PPARγ, possibly via production of PPARγ ligand. Next, PPARγ induce C/EBPα expression and proceed the adipose-conversion synergistically. It is likely that both C/EBPα and PPARγ regulate their expression each other to maintain the differentiate phenotype of adipocyte.

In addition to C/EBPs, PPARs and ADD1, HNF3/*forkhead* family of transcription factor and preadipocyte factor-1 (Pref-1) are also suggested to be involved in the regulation of adipogenesis. Enerback et al. identified two *cis*-regulatory element in enhancer region of LPL and termed LP-α and LP-β. These two element exhibit a high similarity to a consensus binding sequence for HNF-3/*forkhead* family (Enerback et al., 1992). Preadipocyte factor-1 (Pref-1) is a EGF-like protein discovered by differential screening of 3T3-L1 preadipocyte cDNA library. Pref-1 is expressed only in preadipocytes and its constitutive expression in 3T3-L1 cells inhibits the adipose conversion (Smas et al., 1993).

De novo adipogenesis

Acidic fibroblast growth factor (aFGF) (Folkman and Haudenschild, 1980) and basic FGF (bFGF) (Gospodarowicz et al., 1987) are potent inducers of neovascularization. When injected alone subcutaneously into mice, however, bFGF does not induce neovessel formation, probably because the factor was rapidly cleared from the injection site. On the other hand, Passaniti et al. (1992) showed that subcutaneous injection of an extract of basement membrane proteins (Matrigel) supplemented with bFGF into mice reconstitutes the structure of basement membrane in *situ* and supported an intense vascular response. Matrigel is an urea-extract of basement membrane, of which major components such as laminin and type IV collagen are dissociated due to denaturation and can reconstitute the structure of basement membrane after dialysis of urea. Since Matrigel at 4–10 °C is liquid, it can be injected subcutaneously into the mice where it gels as it is warmed to body temperature. This neovascularization could be due to the ability of Matrigel to bind and/or to prevent degradation of FGFs (Folkman et al., 1988) and to stimulate endothelial cell migration. We found that this neovascularization in reconstituted basement membrane creates an ideal microenvironment for *de novo* adipogenesis.

Since adipocytes which form fat pads *in vivo* are surrounded by well developed basement membranes, we first examined the role of basement membrane in adipogenesis by injecting 3T3-F442A preadipocytes subcutaneously into 6 weeks old nude mice over the chest together with Matrigel. Addition of bFGF to the Matrigel + cells at a dose of 1 μg/ml enhanced the adipogenesis. To our surprise, a marked adipogenesis

was also induced by a control injection of Matrigel in combination with bFGF alone (without 3T3-F442A preadipocytes). Except for young mice (see below), injection of Matrigel alone induced poor adipogenesis. 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 (Folkman, 1988) and induced endothelial cells to enter the solidified gel (Passaniti, 1992). This neovascularization together with the basement membrane might create an environment for endogenous adipocyte precursor cells to migrate, proliferate and differentiate.

When the process of *de novo* adipogenesis was followed by preparing histochemical sections of Matrigel plug, neovascularization in Matrigel was completed within 1 week after the injection and well developed blood vessels were formed by a clear endothelium lining. This neovascularization was accompanied by invasion of fibroblast-like cells into Matrigel plug. The differentiation of mature adipocytes was observed along with the neovessels 2 weeks after the injection. Some adipocyte differentiation was observed locally by 1 week after the injection at the periphery of the Matrigel. The population and size of the mature adipocytes increased during 3–5 weeks after the injection and large adipocytes became dominant. 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. In contrast, the plugs formed by injecting Matrigel alone remained quiescent and had few adipocytes, only in the peripheral area on the skin side.

De novo adipogenesis 5 weeks after injecting 100 μl of Matrigel was evident by supplementing with bFGF at only 1 ng/ml and was maximal at 1 μg/ml. Injection of Matrigel into young (1–3 week old) mice also caused intense adipogenesis but the induction did not show clear dependency on supplemented bFGF. Limited examples showed *de novo* adipogenesis in young mice by injection of Matrigel alone. This may be due to either abundant adipose precursor cells present in young animals which responded to the trace of bFGF in the Matrigel preparation, or an active supply of endogenous bFGF to the Matrigel plug from surrounding tissues.

Subcutaneous injections 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 which could be injected subcutaneously 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 masticatory muscle also induced angiogenesis followed by fat pad formation with a less homogeneous population of adipocytes.

In experiments of testing other growth factors, platelet-driven growth factor (PDGF) was as potent as bFGF when co-injected with Matrigel. Despite strong adipogenic effects of growth hormone (GH), 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.

Conclusion

Many lines of evidence show that adipocyte precursor cells distribute widely in the connective tissues of adult body and they have high potential of adipogenesis depending on microenvironment. The hyperplastic formation of adipose tissue in aged animals depending on the dietary condition supports this understanding. Many experiments on rodents have shown that adipocyte precursor cells retain such potential over the life-long period. The fat depots of old mice contain large amount of early markers of adipocyte differentiation (Ailhaud, 1990). A significant population of stromal-vascular cells from subcutaneous fat tissues of old men and women has been shown to differentiate *in vitro* into adipocytes (Hauner et al., 1989).

We have found that such a microenvironment can be created *in situ* by injection of reconstitutive basement membrane, Matrigel. Once neovascularization was enhanced by bFGF, endogenous adipose precursor cells actively migrated into Matrigel plug and differentiated into adipocytes. Invasion of many fibroblast-like cells into 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 subcu-

taneous connective tissues and even in muscle and ear cartilage.

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 (Billings and May, 1987). These include local treatment of the implantation bed with insulin and transplanting small grafts. Partial success was achieved by supplementing the grafts with bFGF (Eppley, 1992). We here showed 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, responsible molecule among basement membrane components needs to be identified.

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