Reconstituted basement membrane potentiates *in vivo* adipogenesis of 3T3-F442A cells

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Abstract

Adipocytes forming fat pad *in vivo* are surrounded by well developed basement membranes. Synthesis of basement membrane is enhanced during *in vitro* differentiation of preadipocyte line. In order to know the role of basement membrane in adipogenesis *in vivo*, we injected 3T3-F442A preadipocytes subcutaneously into nude mice together with or without the reconstituted basement membrane, Matrigel. Histological sections of the fat pads newly formed by injecting the cell alone showed dense population of immature adipocytes and microvessels within 2 weeks and they matured rapidly. In contrast, injection of the cells together with Matrigel showed sparse adipocytes after 2 weeks and they matured slowly over the period of 6 weeks. Quantification of the process by measuring the weight, DNA content, triglyceride content and glycerophosphate dehydrogenase (GPDH) activity of the fat pads showed that injection of the cell alone resulted in early maturation of adipose tissue with fewer adipocytes while the presence of Matrigel decelerated but potentiated the maturation of adipose tissue with 2 fold contents of DNA, triglyceride and GPDH activity. We thus showed that reconstituted basement membrane (Matrigel) supported the survival and maturation of adipocytes.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EHS sarcoma, Engelbreth-Holm-Swarm mouse sarcoma; GPDH, glycerophosphate dehydrogenase; TG, triglyceride

Introduction

Adipocytes are thought to develop from fibroblast-like stem cells widely distributed in connective tissues. Their differentiation begins with the expression of specific enzymes, followed by the accumulation of small lipid droplets which coalesce and enlarge until most of the cell volume is occupied by a single lipid drop with the cytoplasm forming a thin rim (Johnson and Greenwood, 1988). 3T3-L1 and 3T3-F442A are the mouse cell lines having high frequency of adipose conversion *in vitro* (Green and Kehinde, 1975). They are assumed to originate from the stem cells and have committed the monopotent differentiation into adipocytes. Both

cell lines show the morphology of fibroblast at the growing phase, acquire many lipogenic and lipolytic enzymes after the growth arrest at confluence and become sensitive to many hormones (Rosen et al., 1979; Zezulak and Green, 1986). *In vitro* differentiation of 3T3-L1 and 3T3-F442A can not proceed beyond the stage of small lipid droplet deposition and the cells remain to be flattened probably influenced by extracellular matrix protein such as fibronectin (Spiegelman and Ginty, 1983). Subcutaneous injection of 3T3-F442A into nude mice, on the other hand, forms fat pad containing well developed adipocytes (Green and Kehinde, 1979).

Electron microscopic observations of mature

adipocytes *in vivo* (Napolitano, 1963) revealed that the plasma membrane is surrounded by a region which is similar to basement membrane of other tissues. Actually, 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). These show that synthesis and deposition of basement membrane is the key event for the maturation of adipocytes and the *in vitro* culture system can not reproduce such environment.

Matrigel is a mixture of basement membrane proteins extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma with a urea solution and contains type IV collagen, laminin and perlecan as the major components (Kleinman et al., 1987). These proteins renature after dialysis of urea and form self-associated meshwork upon raising the temperature. Since the meshwork resembles the architecture of basement membrane (Yurchenco and Schittny, 1990), Matrigel serves as reconstituted basement membrane. Applications of Matrigel to *in vitro* culture system showed dramatic effects on the maintenance of differentiated phenotypes of various cell lines (Kleinman et al., 1987).

In order to know the role of basement membrane in adipogenesis *in vivo*, we injected preadipocyte 3T3-F442A subcutaneously into nude mice together with Matrigel. Histological sections and quantification of the weight, DNA content, triglyceride (TG) content and glycerophosphate dehydrogenase (GPDH) activity of newly formed fat pads showed that Matrigel supported the survival and maturation of preadipocytes.

Materials and methods

Preparation of reconstituted basement membrane, Matrigel

Matrigel was prepared from EHS sarcoma as described by Kleinman et al. (1986). Briefly, the EHS 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. Two extracts were combined and dialyzed

against 0.05 M Tris-HCl (pH 7.4) buffer containing 0.15 M NaCl.

Cell culture

3T3-F442A cells were kindly provided by Dr. H. Green (Harvard Medical School, Boston, MA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum under atmosphere containing 5% CO₂ at 37 °C.

Injection of 3T3-F442A preadipocytes into nude mice and analyses of transplants

Nearly confluent cultures of 3T3-F442A preadipocytes were harvested by scraping with a rubber policeman. 2×10^6 cells were suspended in 70 μ l of cold DMEM or Matrigel (10 mg protein/ml) and immediately injected into a 6-week-old Balb/c nude mouse which were anesthetized with diethyl ether. Nine mice were used for each group and each mouse was injected subcutaneously over the chest at three sites (70 μ l each) with a 26-gauge needle attached to 1 ml syringe. All eighteen mice were kept with no wound complications. At 2, 4 or 6 weeks after the injection, three mice were sacrificed from each group and the transplants were excised. The newly formed pads were easily distinguished from surrounding tissues and a well-defined plug between skin and muscles could be excised for the preparation of histological sections and biochemical analysis. After weighing the specimens, some were used for the histological analyses and the rest were used for the biochemical analyses. For the latter analyses, the specimens were cut into three equal pieces, rapidly frozen in liquid nitrogen and stored at -80 °C until the assay of DNA content, TG content and GPDH activity.

Histology of the sections

The specimens were fixed overnight in 4% paraformaldehyde in Dulbecco's phosphate buffered saline (pH 7.4), dehydrated and embedded in paraffin wax. Five- μ m-sections were cut, mounted on slides and stained with hematoxylin-eosin. The sections were examined and photographed under a light microscope (Olympus BH-2).

DNA content

DNA content was assayed as described by Kissane and Robins (1958). Briefly, the samples were added

with 0.3 M trichloroacetic acid, sonically disintegrated and centrifuged at 2000 g for 10 min. The precipitates were allowed to stand for 10 min after the addition of 10 μ l of 0.1 N alcoholic potassium acetate. After centrifuging, the precipitates were added with 10 μ l of absolute alcohol and heated at 60 °C for 15 min. After another alcoholic extraction, the precipitates were dried, added with 10 μ l of 2 M 3,5-diaminobenzoic acid dihydrochloride and then heated at 60 °C for 30 min. Fluorescence was determined in a fluorometer by diluting and adjusting the volume of samples with 0.6 M perchloric acid.

TG content and 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 sonically disintegrated and centrifuged at 15 000 g for 5 min. The supernatants were used for the analyses of TG content and GPDH activity. For TG assay, a Triglyceride E-test kit from Wako Chemical CO. LTD. (Osaka, Japan) was used. GPDH activity was assayed as described (Wise and Green, 1979; Ono et al., 1990). One unit of enzyme activity corresponds to the oxidation of 1 nM of NADH/min.

Statistical evaluation

Each experiment was independently repeated once. Statistical analysis was performed with the aid of the statistical software Stat View-J 4.02 for a Macintosh computer. One-way analysis of variance was performed initially. If significance was obtained at the $\geq 95\%$ confidence level, the Fisher's least significant difference procedure was performed.

Results

Gross morphology and histology of newly formed fat pads

When suspension of 3T3-F442A preadipocytes was injected subcutaneously into nude mice together with or without Matrigel, the swelling formed at injection sites regressed within 1 week and flattened pads became palpable 2 weeks after the injection. The pads increased in thickness and co-injection of Matrigel produced more prominent pads over the period of 6 weeks. When dissected, the white pads were

clearly visible and their macroscopic appearance was identical regardless of co-injection of Matrigel.

Histological sections of the fat pads formed 2 weeks after injection of the cells alone showed mature adipocytes distributed among many developing adipocytes with smaller size (Figure 1A). Some extracellular matrixes were deposited around such developing adipocytes. Cluster of adipocytes were delivered with microvessels but denser delivery was observed in the space among the clusters. Injection of the cells together with Matrigel showed sparse adipocytes delivered with fewer microvessels (Figure 1B). Invasive cells, probably related to inflammatory reactions, were observed in the space of Matrigel. In the fat pad formed by the injection of the cells alone, maturation of adipocytes appeared to be fast and large adipocytes predominate 6 weeks after the injection (Figure 1C). In contrast, the size of adipocytes in the pad formed by co-injection of Matrigel was diverse even after 6 weeks (Figure 1D), suggesting that the maturation is still in the process. Most of injected Matrigel was absorbed and replaced by the maturing adipocytes. Histological sections of the fat pads thus suggested that Matrigel decelerated the maturation of adipocytes. Identity of adipocytes observed in the pads was evaluated by staining the lipids with Sudan IV after preparing frozen sections (not shown). TG accumulation and induction of GPDH activity shown in the next section are other lines of evidence for adipocyte differentiation.

Biochemical quantification of newly formed fat pads

Weight of the pads formed by injection of the cells together with Matrigel (hatched columns in Figure 2A) kept increasing over the period of 6 weeks while the pads formed by injection of the cells alone (open columns in Figure 2A) lost the peak weight at 2 weeks during 4–6 weeks. Consequently, co-injection of Matrigel enhanced tissue growth 1.4-fold 6 weeks after the injection.

Regardless of the co-injection of Matrigel, high and almost identical DNA content was observed in the pads 2 weeks after the injection (Figure 2B). The pads formed by injection of the cells alone lost DNA markedly thereafter. DNA content in the pads formed by co-injection of Matrigel also decreased at 4 weeks but restored at 6 weeks. If we can assume the high level of DNA at 2 weeks after the injection to be transient and due to invasive cells of inflammatory reaction, DNA content in the pads formed by co-injection of

Figure 1. Histological sections of the fat pads formed by injection of 3T3-F442A preadipocytes together with or without Matrigel. Some mature adipocytes and many developing cells with smaller sizes were observed in the fat pads formed by injection of the cells alone at 2 weeks (A). Injection of the cells together with Matrigel formed the pads with sparse population of adipocytes at 2 weeks (B). Large adipocytes were predominant in the fat pads formed by injection of the cells at 6 weeks (C). In contrast, the size of adipocytes in the pad formed by injection of the cells together with Matrigel was diverse at 6 weeks (D). Bar scale = 100 μ m.

Matrigel kept increasing over the period of 4–6 weeks and may reflect the proliferation of preadipocytes.

GPDH activity in the pads formed by injection of the cells alone decreased during the period of 4–6 weeks (Figure 2C), suggesting that adipocytes were enervated. In contrast, the pads formed by co-injection of Matrigel maintained high GPDH activity and showed 2-fold activity compared to the pads formed by injection of the cells alone.

TG content in the pads formed by the co-injection of Matrigel increased in a time-dependent manner. On the contrary, TG content decreased in the pads formed by injection of the cell alone (Figure 2D). Consequently, the co-injection of Matrigel produced 2-fold TG content at 6 weeks.

Discussion

3T3-F442A is a preadipocyte line having high potential of adipose conversion (Green and Kehinde, 1975).

In vitro differentiation of this cell line is proved to be an adequate model system of adipogenesis in terms of the specific genes expressed and the sensitivity to various hormones acquired after growth of the cells is arrested at confluence (Rosen et al., 1979; Wise and Green, 1979; Kuri-Harcush et al., 1984; Zezulak and Green, 1986). In addition, 3T3-F442A is able to give rise to normal fat pads when subcutaneously injected into nude mice (Green and Kehinde, 1979). However, the following questions remain to be addressed for this *in vivo* adipogenesis of 3T3-F442A. 1) What is the cue (instead of growth arrest *in vitro*) of the terminal differentiation? 2) How much population of injected preadipocytes can survive and differentiate? 3) What is the role of basement membrane? By studying the time-course of *in vivo* adipogenesis of injected 3T3-F442A preadipocytes together with or without Matrigel, we tried to answer these questions.

Histological sections of the fat pads formed by injection of the preadipocytes alone showed active adipogenesis within 2 weeks. However, DNA content

Figure 2. Quantification of in vivo adipogenesis of 3T3-F442A preadipocytes subcutaneously injected into nude mice together with or without Matrigel. Nine Balb/c nude mice were subcutaneously injected over the chest at three sites with 3T3-F442A preadipocytes of 2×10^6 cells (per injections) suspended in 70 μ l of cold DMEM (open columns) or Matrigel (10 mg protein/ml; hatched columns). The pads formed at three injection sites at 2, 4 and 6 weeks after the injection were excised from three mice each and the weight (A), DNA content (B), GPDH activity (C) and TG content (D) were quantified. The averages are indicated with standard errors. Statistically significant difference with P value less than 0.01 was not found between any combination of the averages.

of the pads decreased rapidly thereafter, suggesting that a large population of the adipocytes became necrotic in the absence of Matrigel. Consistently, the weight and TG content of the pads decreased over the period of 4–6 weeks. These data thus showed that adipocyte differentiation and breakdown of the adipocytes preceded rapidly in the absence of Matrigel. In contrast, histological sections of the fat pads formed by co-injection of Matrigel showed that the initial induction of adipocyte differentiation was slow and the maximal differentiation did not complete even 6 weeks after the injection. The weight and TG content kept increasing and high GPDH activity was maintained. DNA content did not decrease and resulted in 2-fold level of the pads formed by injection of the cells alone.

These showed that Matrigel supported the survival and maturation of 3T3-F442A preadipocytes.

Fundamental questions worth addressing are how Matrigel delayed the cue of adipogenesis and how Matrigel enhanced the ultimate adipogenesis. Concerning the former question, *in vitro* observation that the contact inhibition of cell-growth at confluence is the prerequisite of adipocyte differentiation may give us a hint. Provided with enough space of reconstituted basement membrane, the preadipocytes in the pads formed by co-injection of Matrigel could proliferate actively during early period. This explains the continued increase of DNA content and the low level of initial TG but the ultimate high levels of DNA and TG. Concerning the latter question, the enhanced tu-

morigenicity of Matrigel when injected with tumor cells (Fridman et al., 1990 and 1991) may be a hint. Tumors produced by injection of tumor cells together with Matrigel appeared to be highly vascularized and less necrotic. Likewise, adipocytes in the pads containing Matrigel might be less necrotic and maintain the differentiated characteristics as demonstrated *in vitro* (Hazen et al., 1995).

Matrigel contains trace of growth factors such as TGFs and FGFs with the amount dependent on the preparations (Kleinmann et al., 1993). Thus, our preliminary experiments gave fat pads with fluctuated weight depending on Matrigel preparations, suggesting that trace of growth factors may influence the process. We used a same preparation of Matrigel throughout the study and confirmed that the preparation contained growth factors less than background level by observing a significantly positive effect of addition of 1 ng/ml of basic FGF on the fat pad weight. These suggest that observed effect of Matrigel was mainly due to its major components such as laminins, type IV collagen and perlecan.

Histological sections revealed that most of the injected Matrigel was absorbed and replaced by the mature adipocytes at 6 weeks. This shows that Matrigel is not a permanent material but an absorbable material for the reconstructive surgery. No intensive inflammation reactions were observed in the sections of pads containing Matrigel. Thus, Matrigel is an ideal materials to support the transplantation of differentiated cells for the tissue reconstruction.

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