Revascularization Determines Volume Retention and Gene Expression by Fat Grafts in Mice

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Autologous fat transplantation is a popular and useful technique in plastic and reconstructive surgery. The efficiency and survival of such grafts is predictable in many cases, but there are still issues to be resolved, such as how to improve graft volume retention. To address the issue of volume retention, we studied the effect of revascularization from the recipient on the size and function of adipocytes in fat grafts. Treatment of mice with TNP-470, an angiogenesis inhibitor, reduced blood flow from the recipient into the graft after subcutaneous transplantation of epididymal fat. The weight of transplanted tissues and the size of adipocytes in the grafts were significantly lower in mice treated with TNP-470 (TNP mice) than in control mice. Expression of genes for enzymes related to lipid accumulation was decreased in the grafts of TNP mice compared with control mice. Moreover, the expression of adipocyte-derived angiogenic peptides, VEGF and leptin, was significantly lower in the grafts of TNP mice than in grafts from control animals. The expression of VEGF and leptin by cultured human adipocytes was increased in the presence of conditioned medium from cultured vascular endothelial cells. These results show that the inhibition of the revascularization of fat grafts after transplantation reduces graft volume retention and cellular function. Early and adequate revascularization may be important for both the supply of nutrients and vasoactive interactions between vascular endothelial cells and adipocytes in graft. Exp Biol Med 230:742–748, 2005

Key words: fat transplantation; TNP-470; revascularization; lipid; VEGF; leptin

Fat tissue is an ideal material for use as a permanent soft tissue substitute in plastic and reconstructive surgery (1–3). Free fat grafts from liposuction aspirates are often used as the donor material for soft tissue augmentation. However, knowledge about graft behavior after transplantation remains inadequate. The frequently unpredictable short survival of transplanted fat grafts raises issues about improving volume retention after transplantation. There are many potential immunologic, nutritional, surgical, and anatomic problems that need to be addressed to achieve longer survival of transplanted fat grafts (4, 5).

To address these issues, we have studied the effect of nutritional conditions on graft survival and cell function in fat grafts using nude mice (6). We found that the weight of transplanted tissue and the size of graft cells were dependent on the systemic nutritional status of the recipient. Furthermore, the expression of genes related to lipid metabolism in the transplanted fat grafts was sensitive to the nutritional changes in mice. These results suggested that maintaining the nutritional status of the recipient is important for improving graft survival after fat transplantation.

A recent study has shown that fat tissue mass may be regulated through the vasculature, meaning that the fat tissue mass is sensitive to angiogenesis inhibitors (7, 8). This is because of decreased endothelial cell proliferation and increased apoptosis of fat tissues in treated animals (7) and suggests that the endothelial cells of vessels in fat may be sensitive to angiogenesis inhibitors. In our model of fat tissue transplantation, the revascularization of grafts was observed a few days after transplantation regardless of the calorie intake of the recipient (6). Thus, the revascularization of transplanted fat tissue is not dependent on the nutritional status of the recipient (6). The present study investigated the effect of TNP-470 (9), an angiogenesis inhibitor, on cell size and function in fat tissue grafts after subcutaneous transplantation in nude mice.

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Materials and Methods

Fat Transplantation into Mice. Male ICR mice (for donor, 4 weeks) and male ICR nude mice (CD-1 [ICR]-nu) (for recipient, 4 weeks) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were housed individually with free access to water and standard chow. Donor and recipient mice were injected with 0.5% pentobarbital sodium (0.1 ml/10 g body wt) into the abdominal cavity. Transplant was harvested *en bloc* as each piece of bilateral whole epididymal fat from ICR mice. Minimal skin incision and undermining were performed in bilateral lateral dorsum of recipient mice, and transplant (each piece of epididymal fat) was implanted into the subcutaneous space (6, 10). TNP-470 (Takeda, Osaka; complexed with β-cyclodextrin) was dissolved in saline and administered every day in the abdominal cavity at 0.01 mg/g (body wt) per day for 4 weeks after the transplantation. Before extirpation of the grafts, a flow index of the grafts was assessed using color Doppler ultrasound as described (11). Briefly, the areas of grafts were identified, and then color photographs were taken. In each photograph, the vessels into the areas were identified. The numbers of vessels communicating between the graft and recipient mice were counted for each graft using at least 20 photographs taken for the graft. Their blood flow was estimated as a score classified into 4 grades: 1, 2, 3, and 4 (1 is the slowest and 4 is the fastest). The flow index of a graft was calculated by folding the vessel (only flowing into graft) number and their averaged flow score for each graft.

Evaluation of Transplanted Fat Mass. Four weeks after fat tissue transplantation, transplanted fat tissues were extirpated under anesthesia as described previously (6). Skin incision was performed in square to surround on the transplanted lesion. Undermining was performed to preserve vessels. Mass of extirpated fat tissue was evaluated by appearance both macroscopically and microscopically. The maximal diameter of cells was measured as follows. The maximum diameters of all adipocytes with the presence of a nucleus were measured in a slice stained with hematoxylin using its photographs. The average of the largest 50 diameters were presented as "cell size" of the slice.

Gene Expression in Transplant. Gene expressions in the extirpated grafts were evaluated by reverse transcription polymerase chain reaction (RT-PCR) as previously described (12). Total RNA was isolated with an RNA extraction kit from Qiagen (Tokyo, Japan). To quantify the mRNA levels of hexokinase, fatty acid synthase (FAS), VEGF, and leptin, 2.0 μg of total RNA was reverse-transcribed and amplified using GeneAmp Gold RNA PCR Reagent Kit from Biosystems (Tokyo, Japan) and the specific primers as described below. The amplification was performed for 35 cycles, 95°C for 1 min, annealing 54°C for 1 min, 72°C for 1 min, and a final extension period of 72°C for 8 mins. The suitable condition for the quantification was determined based on the results of linear amplifications of the products in 30, 35, and 40 cycles. Ten microliters of each PCR product was electrophoresed on 2.0% agarose gel and stained with ethidium bromide. The relative signal intensities of the PCR products were determined with luminescent image analyzer LAS-1000 image analyzer (Fuji Photo Film Co., Tokyo, Japan). The mRNA amounts were normalized to levels of β-actin mRNA, which served as endogenous standard.

Primers. Primers synthesized for hexokinase were 5′-GAAGATGGCCAAGGAGGAGC-3′ and 5′-ACGCTTGGCAAAATGGGGGT-3′ (Genbank code NM013820), for FAS were 5′-ATTGGGCACTCCTTGGGAGA-3′ and 5′-CTCAGGGATAGAGGTGCTGA-3′ (Genbank code NM 017332), for VEGF were 5′-GCGGGCTGCCTCGCAGTC-3′ and 5′-TCACCGCCTTGGCTTGTCAC-3′ (Genbank code NM009505.2), for leptin were 5′-GCTGTGCCCATCCAAAAAGT-3′ and 5′-ACTGCCAGTGTCTGGTCCAT-3′ (Genbank code NM008493), and those for β-actin were 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (Genbank code NM007393) according to known cDNA sequence.

Immunohistochemistry. Serial paraffin-embedded sections (10 μm) of extirpated grafts were used for immunohistostaining as described (13). Slides were stained in the presence of 0.1% BSA with rabbit polyclonal IgG against VEGF (sc-507, Santa Cruz, Santa Cruz, CA, 1:100) at 23°C for 1 hr. Biotinylated anti–rabbit IgG secondary antibodies (Promega; San Luis Obispo, CA) were diluted 1:100. The slides were counterstained with hematoxylin. Controls with nonimmune rabbit IgG were conducted in parallel with each immunoassay procedure.

Cell Culture. A block of human abdominal fat tissue was obtained from a 26-year-old man who gave his informed consent for this study. Primary adipocytes were obtained by means of the ceiling culture method reported by Sugihara *et al.* (14). Briefly, 0.5–1 g of abdominal subcutaneous fat was isolated, minced, and digested with Type I collagenase (Nitta Gelatin, Osaka, Japan). The floating layer after centrifugation was seeded into T-25 flasks filled with DMEM supplemented with 10% FBS, and then the flasks were placed upside down and cultured for 2 weeks. The cells attached on the ceiling surface were harvested with trypsin and grown according to standard procedures. The cell appearance after standard procedure in the flask is fibroblastic with small lipid droplets, and the size and number of lipid droplets increased at confluence in the flask at passage 2 or 3. The experiments were performed in these passages.

Measurement of Cytokines. Plasma samples were immediately prepared by centrifugation at 1500 *g* for 15 mins at 4°C. VEGF and leptin levels were measured by ELISA (R&D Systems, Tokyo, Japan) and RIA kits (Linco Research, St. Charles, MO), respectively, as described previously (15).

Statistical Analysis. Values are reported as means ± SD. Statistical analysis was performed with *t* test with differences considered significant at $P < 0.05$ (2 tailed).

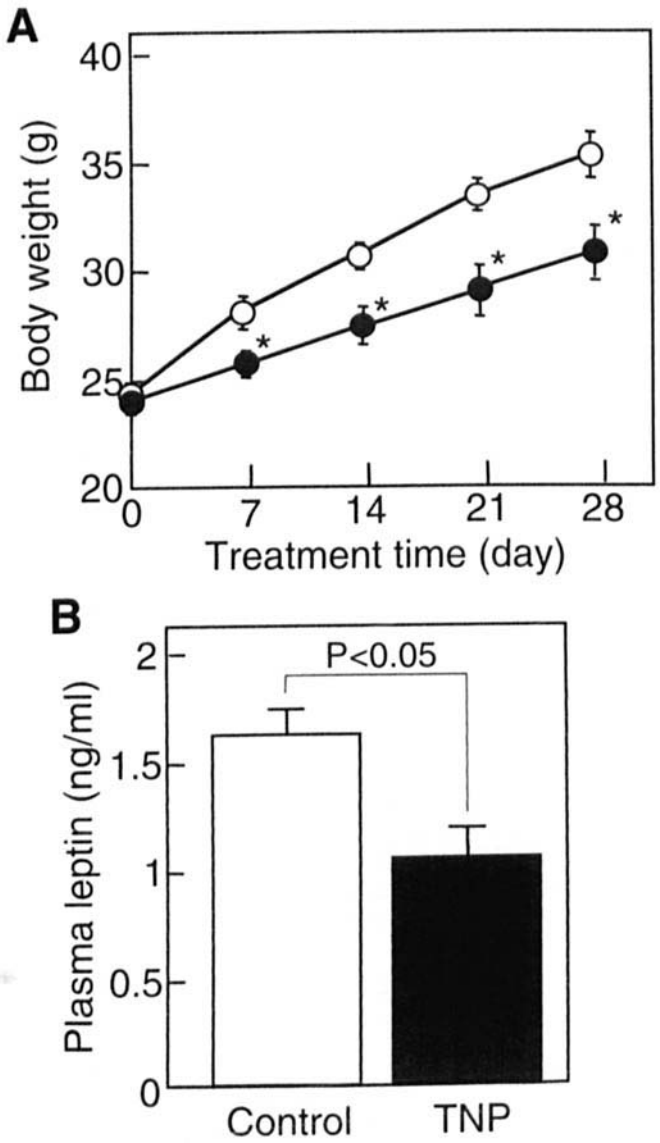

Figure 1. Effect of TNP-470 on fat tissue in mice. Eight-week-old nude mice were divided into two groups ($n = 12$ per group) and treated with 10 mg/kg of TNP-470 (TNP mice, closed circles) and saline (control mice, open circles), respectively, every day after fat tissue transplantation. Body weight of each mouse was measured once a week for 4 weeks after transplantation (A). Plasma leptin level was measured 4 weeks after transplantation (B). Data are mean ± SD ($n = 12$), $^*P < 0.05$.

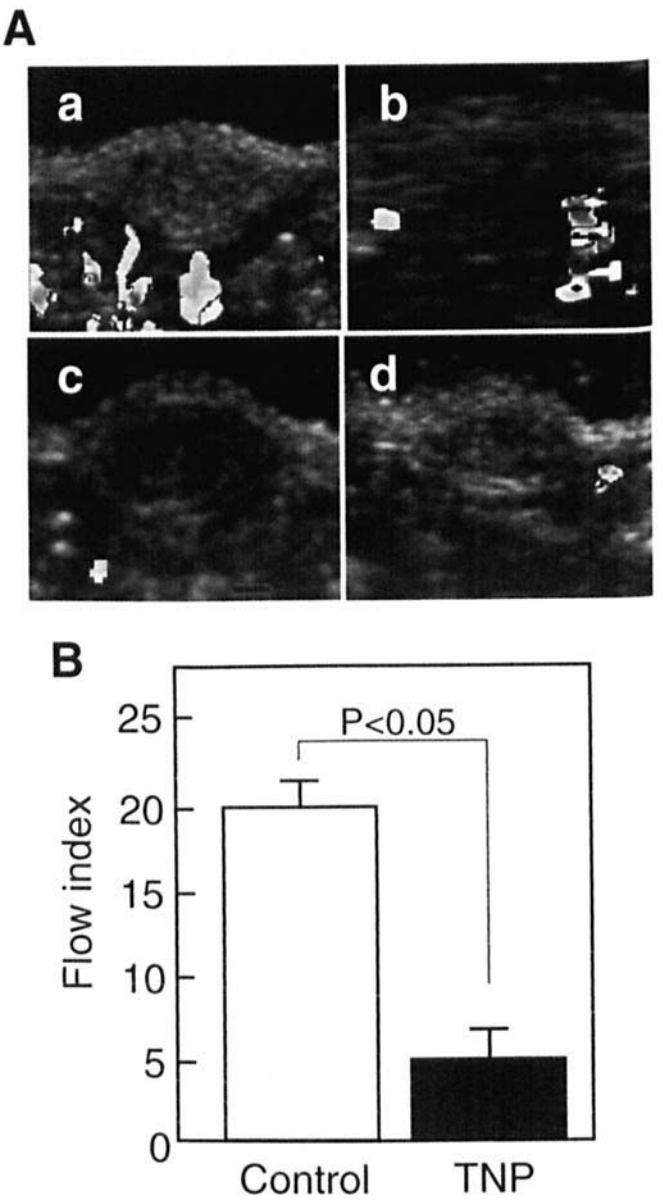

Figure 2. Vascularization of grafts estimated by color-Doppler ultrasonography at 4 weeks after transplantation in mice. The representative photos are presented for grafts in mice (A). The blood flow of vessels for grafts in control (a, b) or TNP mice (c, d) is presented by different gray scales. The flow index was calculated as described in Materials and Methods and compared between TNP mice and control mice (B). Data are mean ± SD ($n = 6$).

Results

TNP-470 Decreases Volume Retention by Fat Grafts. Male mice (4 weeks old) underwent transplantation of pieces of epididymal fat tissue (100–150 mg) into the back and then were fed normal chow with or without TNP-470 (0.01 mg/g) for 4 weeks. The average body weight of both TNP-470–treated mice (TNP mice) and saline-treated mice (control mice) increased over 4 weeks, but there was a significant difference in weight between the groups after 1 week, and the difference persisted (Fig. 1A). No differences of food consumption or serum glucose levels were observed between the two groups (data not shown). To determine whether the poor weight gain of TNP mice was caused by a smaller increase of fat tissue, the plasma leptin level was measured at 4 weeks (Fig. 1B). The average leptin level of TNP mice was significantly lower than that of control mice, suggesting that TNP-470 disturbs lipid accumulation in the fat tissue of mice, consistent with observations previously made in other models (7, 8).

TNP-470 Reduces Graft Blood Flow and Adipocyte Size. The blood flow from the surrounding subcutaneous tissue into the grafts was studied using Doppler ultrasound at 4 weeks after transplantation (Fig. 2A). When the flow index was calculated from the number of vessels and blood flow of each graft, the average flow index of the grafts

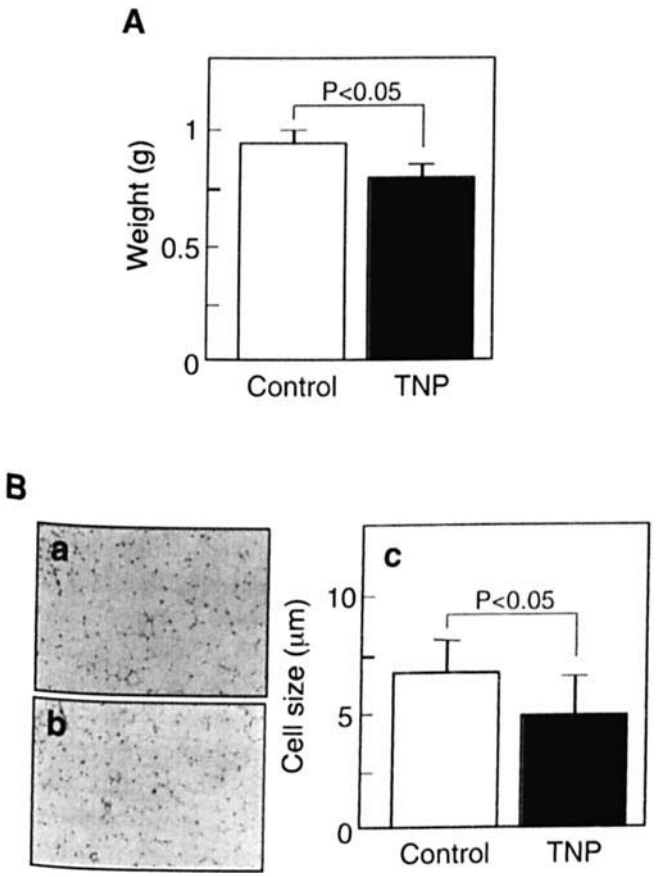

Figure 3. Effect of TNP-470 on weight and cell size in grafts. (A) Comparison of extirpated tissue weights between TNP mice and control mice. Data were presented as the mean ratio of weight in the extirpation and before transplantation. Data are mean ± SD ($n = 6$). (B) Representative microscopic appearance of the extirpated fat tissues from TNP mice (a) or control mice (b). Slices were stained with hematoxylin-eosin. Magnifications are ×40. (C) Comparison of sizes of adipocytes in slices of grafts from TNP and control mice. Data are mean ± SD ($n = 1000$).

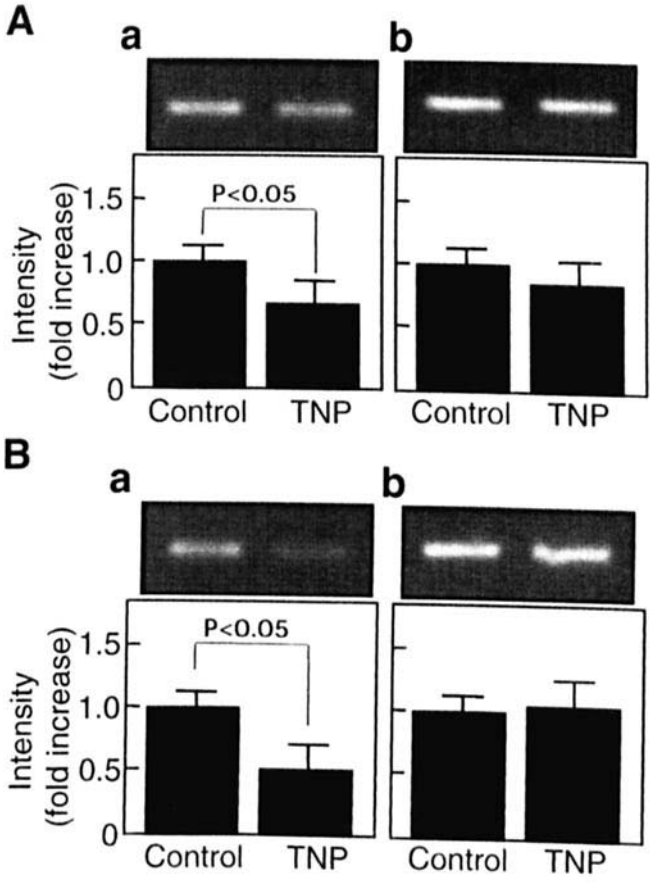

Figure 4. Hexokinase (A) and FAS (B) gene expression in grafts (a) or epididymal fat tissues (b) in TNP or control mice. Total RNA from graft or epididymal fat was prepared and used for cDNA synthesis as described in Materials and Methods. RT-PCR was performed for 35 cycles. One tenth of amplified fragments were used for electrophoresis on a 2.0% agarose gel. Values are the means ± SD ($n = 5$). The Inset shows the result of typical RT-PCR experiment. The intensity of PCR product in the grafts in TNP mice was shown as "fold increase" of that in control mice.

in TNP mice was significantly decreased compared with that for control mice (Fig. 2B).

After harvesting, the weight of the resected grafts was measured. The average weight of grafts removed at 4 weeks after transplantation was significantly lower in TNP mice than in control mice (Fig. 3A). Then the size of adipocytes was analyzed using sections of grafts harvested from the mice (Fig. 3B). The average size of 200 adipocytes in a section stained with hematoxylin-eosin was significantly decreased in TNP mice compared with control mice. These results, together with the results displayed in Figures 1 and 2, suggest that the decrease of vascularity caused by TNP-470 led to impaired volume retention by adipocytes in the grafts.

TNP-470 Reduces the Expressions of Genes Coding Enzymes Promoting Lipid Accumulation. We have previously shown that the expression of genes related to lipid metabolism in transplanted fat tissue is regulated by the systemic nutritional status of recipient mice (6). Therefore, the expression of genes for enzymes involved in lipid accumulation was analyzed in the grafts using RT-PCR (Fig. 4). The hexokinase mRNA level was significantly lower in the grafts of TNP mice than in control mice, whereas the levels in epididymal fat did not differ between the two groups (Fig. 4A). Furthermore, the FAS mRNA level was significantly decreased in the grafts of TNP mice compared with grafts from control mice (Fig. 4B). The marked reduction in the expression of genes related to lipid metabolism suggested that inhibition of revascularization by TNP-470 led to functional changes of adipocytes within the grafts.

Inhibition of Revascularization Reduces VEGF Gene Expression in Grafts. We have shown that the adipocytes express the active form of VEGF, a potent stimulator of angiogenesis, *in vivo* (15). Therefore, the secretion of VEGF from transplanted fat tissue might enhance vasculogenesis from the host tissues around the graft. In the present study, VEGF expression was significantly reduced in the grafts of TNP mice compared with those in control mice, whereas VEGF expression in epididymal fat did not differ between the two groups (Fig. 5A). Furthermore, the

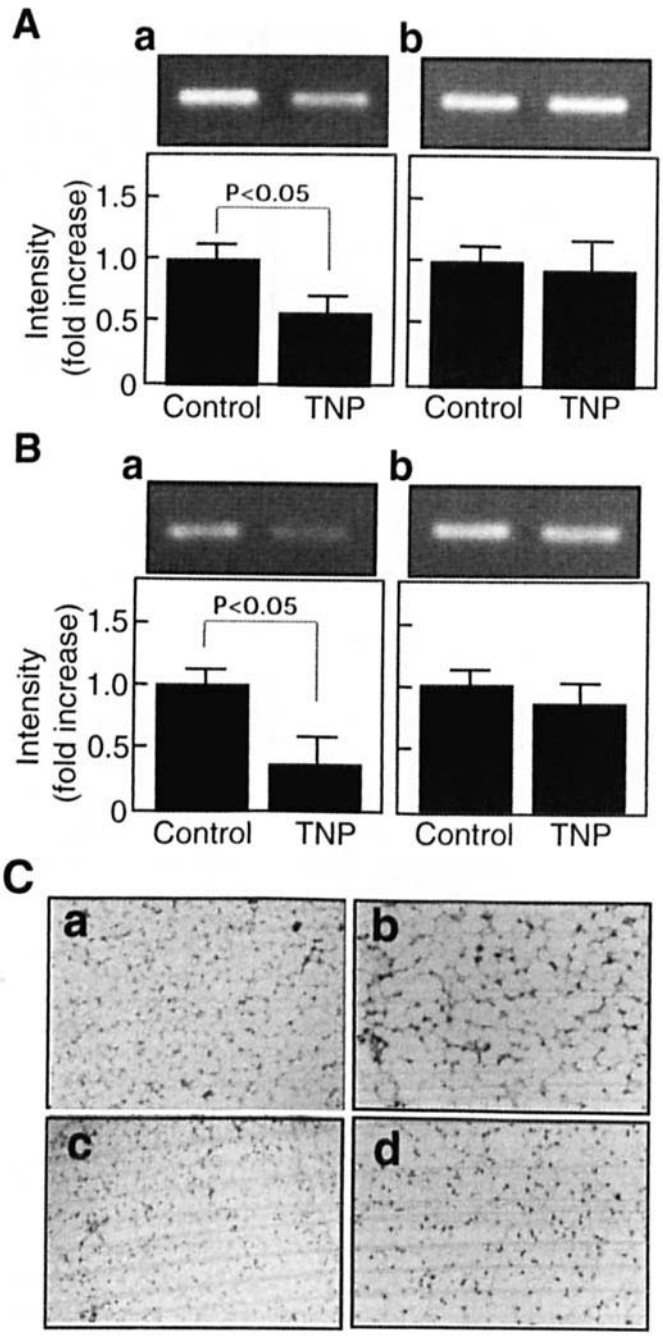

Figure 5. VEGF (A) and leptin (B) gene expression in graft (a) or epididymal fat (b) tissues of TNP mice and control mice. Total RNA from graft and epididymal fat tissues was prepared and used for cDNA synthesis as described in Figure 4. Values are the means ± SD (n = 5). The Inset shows the result of a typical RT-PCR experiment. The intensity of PCR product in the grafts in TNP mice was shown as "fold increase" of that in control mice. (C) Frozen sections of grafts in TNP mice (a and b) or control mice (c and d) were immunohistochemically stained using anti-mouse VEGF antibody. a and c, ×20; b and d, ×100.

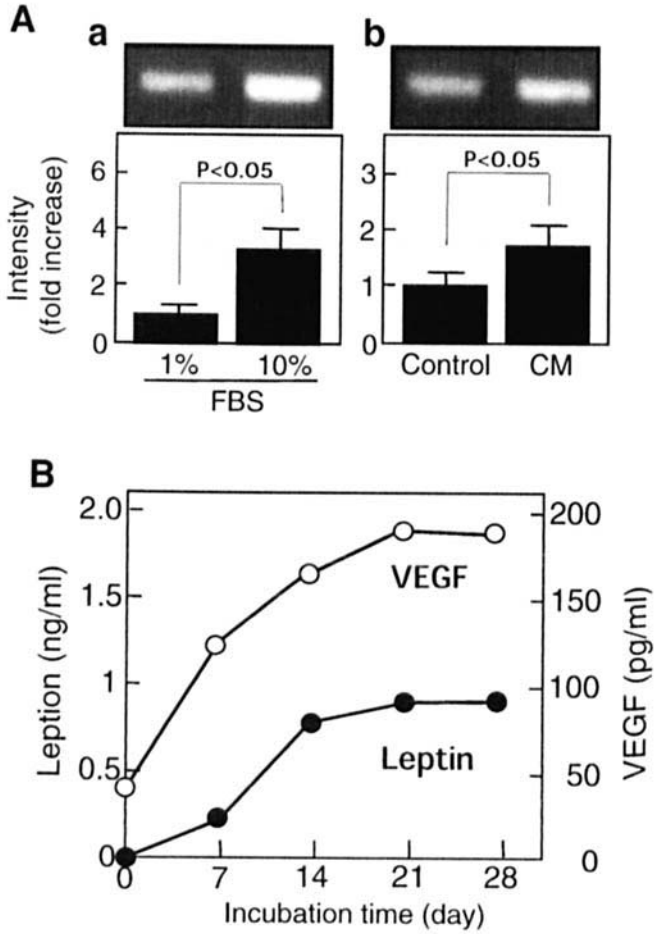

Figure 6. (A) Effects of incubation with FBS (a) or HUVEC-conditioned media in the presence of 1% FBA (b) on VEGF gene expression in human cultured adipocytes. Total RNA from cultured cells was prepared and used for cDNA synthesis as described in Figure 4. Values are the means ± SD (n = 5). The inset shows the result of a typical RT-PCR experiment. The intensity of PCR product in the grafts in TNP mice was shown as "fold increase" of that in control mice. (B) Effect of incubation with HUVEC-conditioned media on the secretion of VEGF and leptin. Conditioned medium was collected for 24 hrs, and the concentrations of VEGF and leptin were analyzed as described in Materials and Methods.

expression of leptin, another angiogenic factor, was significantly reduced in the grafts of TNP mice compared with its expression in control mice (Fig. 5B). Immunohistochemistry using sections of harvested grafts showed that staining for VEGF was decreased in TNP mice compared with control mice (Fig. 5C). Taken together, revascularization of the graft seems to be important for the promotion of cellular function, as well as lipid accumulation, in fat grafts.

Endothelial Cell–Conditioned Medium Induce-VEGF Expression in Cultured Human Adipocytes. Extremely high angiogenic activity has been reported in adipocytes (7, 8, 15). Therefore, we investigated possible interactions between adipocytes and vascular endothelial cells with regard to the above functions of adipocytes in grafts, in addition to the effect on nutritional supply. Addition of serum induced VEGF gene expression dose-dependently in cultured human adipocytes (Fig. 6A). Conditioned medium from HUVEC cultures increased VEGF expression by adipocytes in the presence of 1% FBS. The HUVEC medium also increased VEGF secretion by the adipocytes (Fig. 5B), and the level of VEGF gradually increased by

fourfold after incubation with 1% conditioned HUVEC medium. Leptin was undetectable in the culture medium of human adipocytes, but incubation of adipocytes with 1% conditioned HUVEC medium induced detectable leptin levels. These results indicate that revascularization of fat grafts induces gene expression in adipocytes, possibly mediated by factors secreted from vascular endothelial cells, as well as the increased supply of nutrition from the host tissues around the graft.

Discussion

Transplantation of fat is a useful and common clinical technique in plastic and reconstructive surgery. However, the outcome is variable and seems to depend on the nutritional status of the recipient, the methods of graft preparation, and other factors (3–5). One of the major determinants of the outcome of transplantation is revascularization of the graft from the graft bed. Our previous study using mice with different levels of calorie intake showed that the systemic nutritional status has an important influence on the size and function of adipocytes in subcutaneously transplanted fat (6). Vasculogenesis from surrounding tissues into the graft can be observed early after transplantation (6). This may be because of the high vasculogenic activity of fat tissues, which abundantly secrete cytokines such as VEGF and leptin (15–17). Because of the high vasculogenic ability of fat tissue, subcutaneous injection of VEGF into the graft region did not significantly improve the efficiency of transplantation in mice fed a poor diet (6). In agreement with these results, Rupnick *et al.* showed that adipose tissue is active in promoting vascularization and is sensitive to angiogenesis inhibitors, so that the fat tissue mass can be regulated by its vasculature (7). On the basis of recent findings, we studied the influence of revascularization on the size and function of adipocytes in fat tissue grafts by treating mice with an angiogenesis inhibitor, TNP-470 (18). We further analyzed the effect of vasculogenesis on gene expression and cytokine secretion by cultured human adipocytes, focusing on the interaction between adipocytes and vascular endothelial cells.

Treatment of mice with TNP-470 causes dose-dependent, reversible weight reduction and loss of adipose tissue (7, 8). Marked vascular remodeling has been detected in adipose tissue on histological examination, which has revealed decreased vascular endothelial cell proliferation and increased apoptosis in treated mice (7). In our study, the weight of TNP mice was significantly lower than that of control mice, although the weight of both groups increased during the treatment period. Measurement of leptin level at 4 weeks after transplantation indicated that the weight difference between the two groups was mostly caused a difference of fat mass, as shown in *ob/ob* mice (7,8). The specificity of TNP-470 makes it suitable to study the effect of revascularization on fat tissue transplantation because of the high dependence of graft on revascularization for survival and cellular activity.

Revascularization of transplanted fat tissue has been studied quantitatively in hamsters using a transparent titanium dorsal skinfold chamber (19). Revascularization reached the center of the graft on Day 3 after transplantation (19), in agreement with our previous observation (6). In the present study, blood flow was detected in the grafts of TNP mice, but the flow index was obviously decreased compared with that of control mice, suggesting that nutritional supply to the graft might not have been enough for the maintenance of adipocyte size and function. Decreased expression of genes for lipogenic enzymes and cytokines was evident in the grafts of recipients treated with TNP-470 compared with epididymal fat. Thus, adipocyte function seems to be largely dependent on the activity of vasculogenesis. These results need further analysis using an autologous or syngeneic model in the future because of the evaluation of involvement of TNP-470 in the immune systems.

Adipocytes secrete several cytokines, some of which are important for the homeostasis of glucose and lipid metabolism and/or maintenance of the vessel wall (16, 17). Among them, VEGF is a potent angiogenic peptide that is secreted by adipocytes in its active form, and the circulating VEGF level is positively correlated with the fat mass in humans (15). Leptin is another peptide that promotes angiogenesis by increasing the proliferation of vascular cells (16, 20). The expression of these genes and secretion of active peptides in the grafts were sensitive to the inhibition of vasculogenesis in our model. On the basis of the observation that expression and secretion of these factors were increased in cultured human adipocytes by conditioned medium from endothelial cells, the potent angiogenic activity of adipocytes is suspected to be partly regulated by interaction with the endothelial cells of peripheral vessels. To enhance the angiogenic activity of graft adipocytes, early and extensive vasculogenesis might be required after transplantation.

In summary, graft vasculogenesis is important for efficient cellular function in transplanted fat tissue. Inhibition of vasculogenesis by an angiogenesis inhibitor significantly reduced graft volume retention and the function of adipocytes in the grafts. Factors derived from vascular endothelial cells might be important for the high angiogenetic activity of grafted adipocytes. Further molecular studies on the regulation of angiogenic factors in vascular endothelial cells as well as adipocytes may be needed to improve the efficiency of fat tissue transplantation.

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