JOURNAL OF SURGICAL RESEARCH **67,** 147–154 (1997)
ARTICLE NO. JR964983

Vascular Endothelial Growth Factor Is the Major Angiogenic Factor in Omentum: Mechanism of the Omentum-Mediated Angiogenesis

QING-XUE ZHANG, PH.D., CHRISTOPHER J. MAGOVERN, M.D., CHARLES A. MACK, M.D., KURT T. BUDENBENDER, B.S., WILSON KO, M.D., AND TODD K. ROSENGART, M.D.[1]

Department of Cardiothoracic Surgery, The New York Hospital–Cornell University Medical College, 525 E. 68th Street, F-2103, New York, New York 10021

Submitted for publication July 22, 1996

Omentum has been used clinically to promote wound healing and to stimulate the revascularization of ischemic tissues. The biologic mechanism responsible for these effects has, however, not yet been defined. A number of polypeptide growth factors that possess potent angiogenic properties have recently been identified, and we therefore sought to determine whether one of these growth factors might be responsible for the angiogenic properties of the omentum. The levels of vascular endothelial growth factor (VEGF) protein in a number of rat tissues and organs were analyzed by Western and enzyme immunoassay analysis. Because omentum was found to have the greatest VEGF concentrations of the tissues examined, antibody neutralization, transcription inhibition assays, and Northern blot analysis were performed under hypoxic and normoxic conditions on tissue extractions and primary tissue cultures of omentum to further characterize the functional significance of VEGF expression in these tissues. The omentum demonstrated the highest VEGF secretion rate as well as the highest concentration of VEGF protein of the various rat tissues and organs examined. Fractionation studies of the omentum furthermore demonstrated that omental adipocytes, rather than the stromal–vascular cells, were the primary source of VEGF protein. An endothelial cell mitogenic assay showed that a major portion of the mitogenic activity of heparin-binding proteins and conditioned media derived from omentum was abolished by VEGF antibody. Additional studies with the transcription inhibitor actinomycin-D furthermore demonstrated that the VEGF gene was continuously transcribed in the rat omental adipocytes. Incubation of the omental adipocytes under hypoxic conditions induced approximately a 1.7-fold increase in VEGF protein expression, which was abolished by actinomycin-D. Northern blot analysis demonstrated that hypoxia resulted in upregulation of the VEGF mRNA in the hypoxia-cultured omental adipocytes, suggesting that the augmentation of VEGF expression in omental adipocytes by hypoxia occurs at the transcriptional level. These data suggest that VEGF is the major angiogenic factor produced by omentum and possibly underlies the mechanism of omentum-induced angiogenesis. Augmented expression of VEGF by omental cells under hypoxic conditions may furthermore reflect the mechanism responsible for enhancing the angiogenic activity of omentum in the setting of ischemia.

[1] Supported in part by an American Heart Association Grant-in-Aid (94066).

INTRODUCTION

Angiogenesis, the formation of new capillaries and blood vessels, plays a key role in normal physiological processes such as growth and wound healing and in the progression of pathological conditions such as tumorogenesis and atherosclerosis [1]. The angiogenic processes are thought to be regulated by a balance between stimulatory angiogenic factors, including fibroblast growth factor (FGF), platelet-derived growth factor, and vascular endothelial growth factor (VEGF), and inhibitory angiogenic factors such as angiostatic steroids and angiostatin [2].

The angiogenic property of omentum has been documented since 1585. Ever since these initial observations, omentum has been used clinically to promote revascularization and healing of compromised or ischemic organs and tissues, including the myocardium and intestinal and bronchial anastomoses [3–5]. The improved healing induced by omentum has been demonstrated to be the result of new blood vessel formation, or angiogenesis. Despite these clinical applications, the angiogenic factor(s) in omentum has not yet been identified.

Adipose tissue including the omentum, once conceived of as a relatively inactive biological substrate, in fact serves as the primary site for a number of critical biologic functions including fatty acid metabolism and lipoprotein lipase synthesis [6]. Adipose tissue is also capable of producing and secreting substances such as adipsin and tumor necrosis factor-α that have significant regulatory functions [7]. Because adipose tissue serves as an important conduit for the vasculature, it

0022-4804/97 $25.00

is conceivable that the angiogenic properties of adipose tissue may modulate the growth of the vasculature in a paracrine mode. Monobutyrin, a lipid angiogenic factor isolated from the adipocyte-conditioned medium, has been considered a potential candidate responsible for the angiogenic activity of adipose tissue. Monobutyrin is not, however, mitogenic for endothelial cells and only stimulates endothelial cell migration [8, 9]. Therefore, other vascular endothelial cell mitogens may be involved in adipose-tissue-mediated angiogenic activity.

VEGF is a heparin-binding glycoprotein that is a potent, endothelial cell-specific mitogen and angiogenic growth factor *in vitro* and *in vivo* [10]. VEGF has been found to be widely expressed at the transcriptional level by a number of different tissues and by a variety of cell lines [11–13]. However, quantification of the distribution of VEGF protein in an intact animal has not to date been systematically determined. We therefore sought to characterize the distribution of VEGF at the protein level, and to determine whether VEGF was an important constituent of omentum, potentially responsible for the angiogenic potential of omentum.

MATERIALS AND METHODS

Protein extraction and immunoblotting analysis. Rat tissues and organs were harvested from 4-week-old rats (Sprague–Dawley, 200–250 g) sacrificed by pentobarbital overdose. The animals were treated according to the National Research Council's animal care regulation with the approval of the institutional review board. Biopsies were taken of various organs and tissues as indicated. The biopsies were extensively washed with saline and then electrically homogenized in protein extraction buffer (10 m*M* Tris, pH 8.0, 0.14 *M* NaCl, 0.025% sodium azide, 2% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 1 μM leupeptin) and the homogenates were subsequently centrifuged for 10 min (13,500 rpm, 4°C). Total protein was quantified using the BCA protein assay kit (Pierce Chemicals, Rockford, IL) following the manufacturer's instruction. Equal amount of total proteins (100 μg) from each specimen were incubated with pre-equilibrated heparin–Sepharose CL-6B beads (Pharmacia, Sweden) in 1 ml equilibrate buffer (10 m*M* Tris, pH 7.2, 50 m*M* NaCl, and 1 m*M* PMSF) at 4°C for 2 hr. The heparin–Sepharose beads were then collected by centrifugation (5000 rpm, 2 min), washed twice with the equilibration buffer, and suspended in the loading buffer (125 m*M* Tris, pH 6.8, 0.5% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.001% bromophenol blue), and the bound protein was subsequently eluted from the beads by boiling for 5 min. The proteins were separated on a SDS–12% polyacrylamide gel, electrically transferred to nitrocellulose membrane, and immunoblotted overnight at 4°C with an affinity-purified polyclonal VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 0.5 μg/ml. The blot was washed, probed with an alkaline phosphatase goat anti-rabbit IgG secondary antibody, and processed with alkaline phosphatase detection reagents. Purified recombinant human (rh) $VEGF_{165}$ (Upstate Biotechnology, Upstate, NY) was used as a positive control in the immunoblotting analysis.

Enzyme immunoassay. A competitive VEGF enzyme immunoassay (EIA) kit (CytImmune Sciences, College Park, MA) was used to quantify the level of VEGF in tissue samples and in the conditioned cell media. Fifty micrograms of the total tissue protein or 50 μl of the conditioned cell media were used in the EIA. A standard curve was included every time and triplicate wells were conducted for each specimen.

Obtaining heparin-binding protein from omentum. Heparin-binding proteins from omentum were obtained by heparin–Sepharose affinity chromatography [14]. Briefly, total protein extracted from the omentum was diluted with 10 volumes of equilibrate buffer, loaded onto a 2-ml heparin–Sepharose column. The column was then washed with excess equilibrate buffer and eluted with 2 ml elution buffer (equilibrate buffer plus 0.9 *M* NaCl). The elutes were dialyzed against the equilibrate buffer overnight at 4°C. The amount of VEGF in the dialyzed eluent was quantified by EIA and used in the endothelial mitogenic assay.

Isolation and culturing of omental cells. Omental adipocytes and the vascular–stromal cells were isolated as described [15]. For analysis of the distribution of VEGF, protein extracts were obtained by lysis of the cells with the protein extraction buffer. Two hundred micrograms of the total protein from each fraction was immunoblotted as described above. For culturing the omental adipocytes, the cells from 1 g tissue were incubated with 2 ml medium (DMEM plus 1 m*M* PMSF) in a CO_2 incubator at 37°C with or without cycloheximide (50 μg/ml) or actinomycin-D (10 μg/ml). The conditioned culturing media were collected 24 hr after the incubation and used for immunoblotting, EIA quantification, and the endothelial mitogenic assay. Since pituitary has been considered to be a major site of VEGF production, cells isolated from pituitary as described [16] were also included for the purpose of comparison.

Culturing omental cells under hypoxia. Omental cells were isolated as described above. Briefly, 1×10^4 cells were suspended in 1 ml serum-free medium with or without actinomycin-D (10 μg/ml) and cultured in a six-well plate. The plate was then put into an air-tight chamber continuously infused with premixed gas mixture (1% O_2, 5% CO_2, 94% N_2; Matheson Gas Products, Inc., New York, NY) at a flow rate of 3 L/min. The conditional medium was collected 18 hr later and filtered through a syringe filter with 0.2 μm pore size. The amount of secreted VEGF in the filtered conditional medium was quantified by VEGF EIA. Lactate dehydrogenase activity in the conditional medium was also conducted to monitor the viability of the cells as described below.

Lactate dehydrogenase assay. Lactate dehydrogenase (LDH) activity was measured to determine the viability of the primary omental cells during the period of incubation. A LDH assay kit (Sigma Chemicals, St. Louis, MO) was used according to the manufacturer's instructions. The LDH activities both in the cells and in the conditioned media were measured.

Endothelial mitogenic assay. Human umbilical vein endothelial cells (HUVEC; gift from E. Jaffe, Cornell University Medical College, New York, NY) were used in the assay. Briefly, 2×10^4 cells were plated in each well of a 12-well plate with the assay medium (M199, 10% fetal bovine serum, 50 μg/ml streptomycin/penicillin) and incubated overnight for attachment. The heparin-binding protein from omentum and the conditioned media of the omental adipocytes with or without the VEGF neutralizing antibody (10 μg/ml) was added to the cells. The cells were incubated for 5 days and the number of cells in each well was counted subsequently. Triplicate wells were counted for each treatment.

Northern blot analysis. Total RNAs were extracted from the normoxia-cultured omental adipocytes, the hypoxia-cultured omental adipocytes, and the hypoxia-cultured omental adipocytes treated with actinomycin-D at 10 μg/ml. Equal amounts of the total RNAs (20 μg) were fractionated on a 1% agarose–formaldehyde gel, transferred to a Nytran membrane, and UV crosslinked to the membrane. The cDNA probe for the VEGF was generated by excising the cDNA insert from the plasmid containing the VEGF cDNA. The insert was separated on an agarose gel, extracted, purified, and labeled with [^{32}P]dCTP by the random primer labeling technique. Denatured probes (1×10^6 cpm/ml) were hybridized overnight at 45°C with the prehybridized Nytran membrane containing the RNA. The blot was then removed, washed at 65°C, air-dried, and autoradiographed.

Statistical analysis. Student's *t* test was used to analyze the data. Significant difference among the various treatments was indicated by a *P* value less than 0.05. All data represent the mean ± SE.

RESULTS

Abundant Expression of VEGF Protein in Omentum

The expression of VEGF at the protein level was characterized in a number of different tissues by immu-

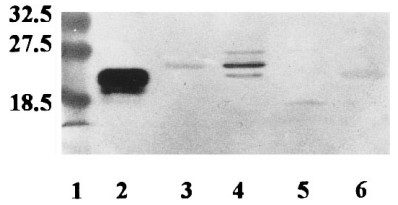

FIG. 1. Western blot of the total proteins from rat tissues and organs. The total proteins from 5 mg of the tissues or organs were immunoblotted as described under Materials and Methods. A VEGF expression product was not detectable by Western blot for other biopsied tissues and organs included in Table 1; the corresponding lanes therefore are not shown. Lane 1, prestained protein marker; 2, rh-$VEGF_{165}$; 3, inguinal adipose tissue; 4, omental adipose tissue; 5, retroperitoneal adipose tissue; 6, pituitary.

noblotting analysis and EIA. The highest levels of VEGF were noted by Western blot analysis in the omentum (Fig. 1). EIA quantification confirmed that the level of VEGF protein was greatest in omentum, and that relatively high levels of VEGF protein were also present in pituitary and inguinal adipose tissue (Table 1). Levels of VEGF protein detected in older (4-month) rats were similar to those seen in these 4-week rats, and omentum possessed the highest levels of VEGF protein of tissues examined in rabbits and dogs (data not shown), suggesting that these observations were neither age nor species specific.

Cellular Localization of VEGF Protein in Omentum

Omentum consists of adipocytes and vascular–stromal cells, such as vascular endothelial cells, smooth muscle cells, mast cells, and macrophages. To determine the source of the VEGF protein in omentum, omental adipocytes were isolated and separated from the vascular–stromal cells based on the difference in densities of the two groups of cells. Immunoblot analysis of protein extracts from *ex vivo* omental adipocytes and vascular–stromal cells demonstrated that VEGF could only be detected in the adipocytes and not the vascular–stromal cells (Fig. 2A). EIA quantification showed that approximately 97% of the VEGF protein found in omentum was associated with the omental adipocyte fraction (Fig. 2B).

TABLE 1

VEGF Protein Levels in Rat Tissues and Organs

Tissue or organ	VEGF (pg/mg tissue)
Omentum	883.93 ± 14.47
Pituitary	106.13 ± 7.08
Inguinal adipose tissue	87.20 ± 6.54
Hypothalamus	55.30 ± 4.57
Tongue	52.69 ± 10.54
Skeletal muscle	32.32 ± 11.07
Eye	30.91 ± 1.07
Lung	24.55 ± 7.08
Epididymis adipose tissue	20.51 ± 1.94
Plasma	14.61 ± 2.04
Brain	8.13 ± 2.14
Retroperitoneal adipose tissue	7.68 ± 2.69
Kidney	6.26 ± 1.39
Heart	5.87 ± 3.32
Blood cells	5.60 ± 1.12
Liver	4.28 ± 0.86
Pancreas	2.79 ± 0.69
Brain stem	1.45 ± 0.48
Spleen	0.94 ± 0.31

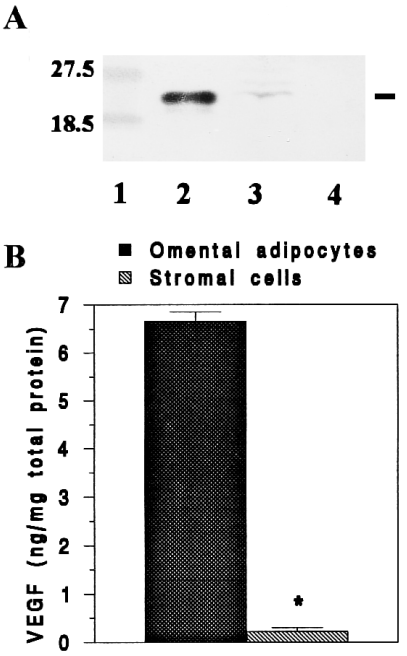

FIG. 2. Cellular localization of VEGF protein in the omental cells. (A) Western blot of the protein extracts from the adipocytes and the vascular–stromal cells of the omentum. Adipocytes and the vascular–stromal cells of the omentum were separated and the total proteins were extracted as described under Materials and Methods. 200 μg of the total proteins from each fraction was immunoblotted. Lane 1, prestained protein marker; 2, rh-$VEGF_{165}$; 3, adipocytes; 4, vascular–stromal fraction. (B) Histogram showing the relative distribution of VEGF protein in the omental cells. Adipocytes and the vascular–stromal cells of the omentum were separated and the total proteins were extracted as described under Materials and Methods. VEGF protein per 50 μg of total protein was quantified by EIA. Three wells were assayed for each specimen. *$P < 0.05$.

VEGF Secretion by Omental Cells

Because several isoforms of VEGF are known to be secreted, the media conditioned by primary adipocytes were assayed by EIA to determine whether VEGF protein was in fact secreted by these cells. Since pituitary was also a major source of VEGF, pituitary cells were also isolated and similarly analyzed. Of the cell types assayed, the highest rate of VEGF secretion was noted for adipocytes derived from omental tissue, with an intermediate rate noted for cells derived from pituitary (Table 2). Immunoblot analysis of the conditioned media of the omental adipocytes demonstrated a single band corresponding to the $VEGF_{165}$ protein marker (Fig. 3).

Neutralization of the Mitogenicity of Omentum by VEGF Antibody

VEGF antibody was utilized to determine whether VEGF protein was an important constituent of the mi-

TABLE 2

Rate of VEGF Secretion by Various Rat Tissues

Tissue	Secretion rate (pg/g tissue/hr)[a]
Omentum	362.51 ± 14.63
Inguinal adipose tissue	91.53 ± 7.57
Pituitary	53.34 ± 5.08
Epididymis adipose tissue	18.61 ± 1.32
Retroperitoneal adipose tissue	6.45 ± 1.33

[a] Cells from rat adipose tissues and pituitary were isolated and cultured as described under Materials and Methods. The conditioned media were collected 24 hr later and the VEGF protein in the media was quantified by EIA. Three wells were assayed for each specimen. The secretion rate was determined by the total VEGF protein secreted during the period of incubation divided by the time.

togenic activity of the heparin-binding proteins isolated from omentum and the medium conditioned by primary omental adipocytes. In order to perform this analysis, endothelial cell growth assays were performed. The results demonstrated that a major portion of the mitogenic activity of the heparin-binding proteins isolated from omentum and the medium conditioned by primary omental adipocytes was abolished by the VEGF antibody (Fig. 4), confirming, respectively, that VEGF is a critical endothelial cell mitogen in omentum and in omental cell secretions.

Regulation of VEGF Expression in the Omentum

To examine whether the VEGF protein secreted from the primary omental adipocytes was a preexisting, cell-associated growth factor or represented newly synthesized growth factor, primary omental adipocyte cultures were incubated with inhibitors of transcription (actinomycin-D) and protein synthesis (cycloheximide). Each inhibitor significantly reduced ($P < 0.05$) the VEGF protein levels in the conditioned media (Fig. 5), indicating that the secreted VEGF protein was newly synthesized. In order to monitor the potential toxicity induced by these inhibitors, the viability of the cultured adipocytes in these studies was confirmed both visually and by LDH assay. Less than 1% of the intracellular

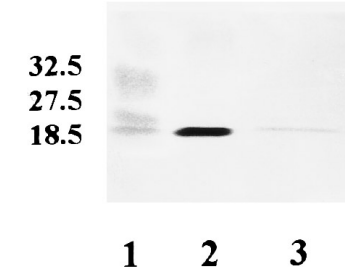

FIG. 3. Western blot of omental adipocyte-conditioned medium. The omental adipocytes were separated and cultured as described under Materials and Methods. The conditioned media were collected 24 hr later. 100 μl of the medium was precipitated by heparin–Sepharose and immunoblotted. Lane 1, prestained protein marker; 2, rh-VEGF$_{165}$; 3, omental adipocyte-conditioned medium.

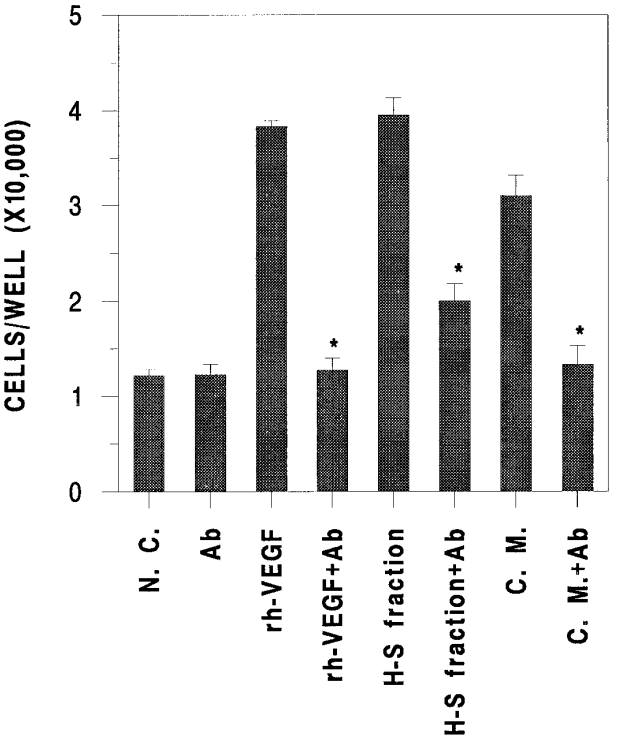

FIG. 4. Bar graph showing the result of the endothelial mitogenic assay. HUVEC cells were plated at 2×10^4 cells/well in 12-well plates and allowed to attach overnight. Then, 2 ng/ml rh-VEGF, 2 ng/ml rh-VEGF plus 10 μg/ml VEGF-neutralizing antibody (rh-VEGF + Ab), heparin-binding proteins of the omental adipose tissue (H-S fraction), heparin-binding proteins of the omental adipose tissue plus VEGF-neutralizing antibody (H-S fraction + Ab), omental primary adipocyte-conditioned media (C. M.), or omental primary adipocyte-conditioned media plus VEGF-neutralizing antibody (C. M. + Ab) was added to the media. The cells were incubated for 5 days and counted. Three wells were assayed for each treatment. *$P < 0.05$ vs the corresponding treatment without the neutralizing antibody. N. C., negative control; Ab, antibody only.

LDH enzymatic activity was detected in the conditioned media, indicating that cell death and release of intracellular proteins did not significantly contribute to VEGF concentrations in the media.

Induction of VEGF Expression in the Omentum by Hypoxia

We examined whether hypoxia would enhance the expression of VEGF by omental cells as further evidence that VEGF is a regulated protein, potentially responsive to stresses such as hypoxia and ischemia for which VEGF-mediated angiogenesis would be an important homeostatic mechanism. In these studies, incubation of the primary omental adipocytes under hypoxic conditions resulted in approximately a 1.7-fold increase in VEGF protein secretion, which was inhibited by the transcription inhibitor actinomycin-D (Fig. 6). Northern blot analysis furthermore demonstrated that the VEGF-specific mRNA expression was induced by hypoxia, and that actinomycin-D also abolished this response (Fig. 7), indicating that this regulation occurred at the transcriptional level.

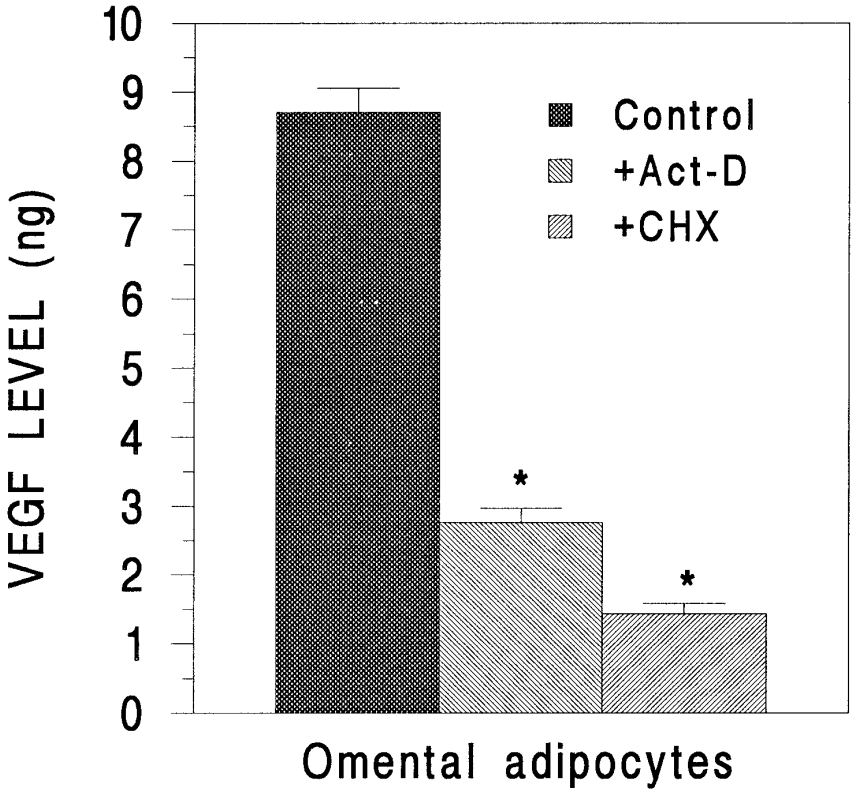

FIG. 5. Effects of transcription inhibitor and protein synthesis inhibitor on the expression of VEGF protein by the primary omental adipocytes. Primary omental adipocytes were isolated and cultured in the presence of either actinomycin-D (Act-D, 10 μg/ml) or cycloheximide (CHX, 50 μg/nl). The conditioned media were collected 24 hr later. VEGF protein in the media was quantified by EIA. Three wells were assayed for each specimen. The data represent the amount of VEGF protein produced by the omental adipocytes from 12 g omentum tissue in 24 hr. *$P < 0.05$ vs control.

DISCUSSION

Expression of VEGF has previously been investigated in different tissues and organs by several investigators. These studies primarily examined VEGF expression at the transcriptional level, however, and because protein is the most biologically relevant product of gene expression, we analyzed the distribution of VEGF in rat tissues and organs as a protein, rather than as an mRNA product. Abundant VEGF mRNA expression, equal to that of several other organs, has previously been noted in epididymal adipose tissues by Claffey *et al.* [17]; other adipose tissues, such as omentum, were not examined in these previous studies. Similarly, adipose tissues were not included in other studies by Berse *et al.* [12] and Monacci *et al.* [13] examining VEGF distribution, and therefore the current finding that the greatest VEGF expression is found in omentum cannot be compared to these previous studies. In contrast, the high levels of VEGF mRNA expression previously noted in organs other than adipose tissue, including kidney and liver, were not associated with corresponding high levels of VEGF protein in these organs in the current study. It may be implied from these data that VEGF expression may be significantly regulated at the posttranscription level. In fact, posttranscriptional regulation has been widely found to regulate eukaryotic gene expression [18], and may explain this discrepancy. Such considerations highlight the need to quantify the biological effects of gene expression at the protein as well as at the mRNA level.

In this study we have analyzed the expression of VEGF protein by immunoblot analysis and, in a more quantified manner, by EIA studies. Both immunoblotting analysis and EIA quantification in the current study demonstrated that omentum contained concentrations of VEGF protein that were greater than that of epididymal adipose tissue or other high VEGF concentration tissues previously identified. It must again be emphasized, however, that VEGF protein concentrations in various tissues may represent the steady-state level of VEGF protein expression in these tissues and are not translatable into levels of VEGF mRNA expression. On the other hand, the failure to detect the presence of VEGF proteins in tissues and organs except the omentum, inguinal adipose tissue, and the pituitary gland by immunoblotting analysis may be due to the low sensitivity of this method, as described in this report. The greater sensitivity of EIA complements immunoblotting in further defining the tissue distribution of VEGF protein expression.

The significant angiogenic potential of omentum, demonstrated in a number of clinical applications and in other mammals such as rabbit, dog, and monkey [19,

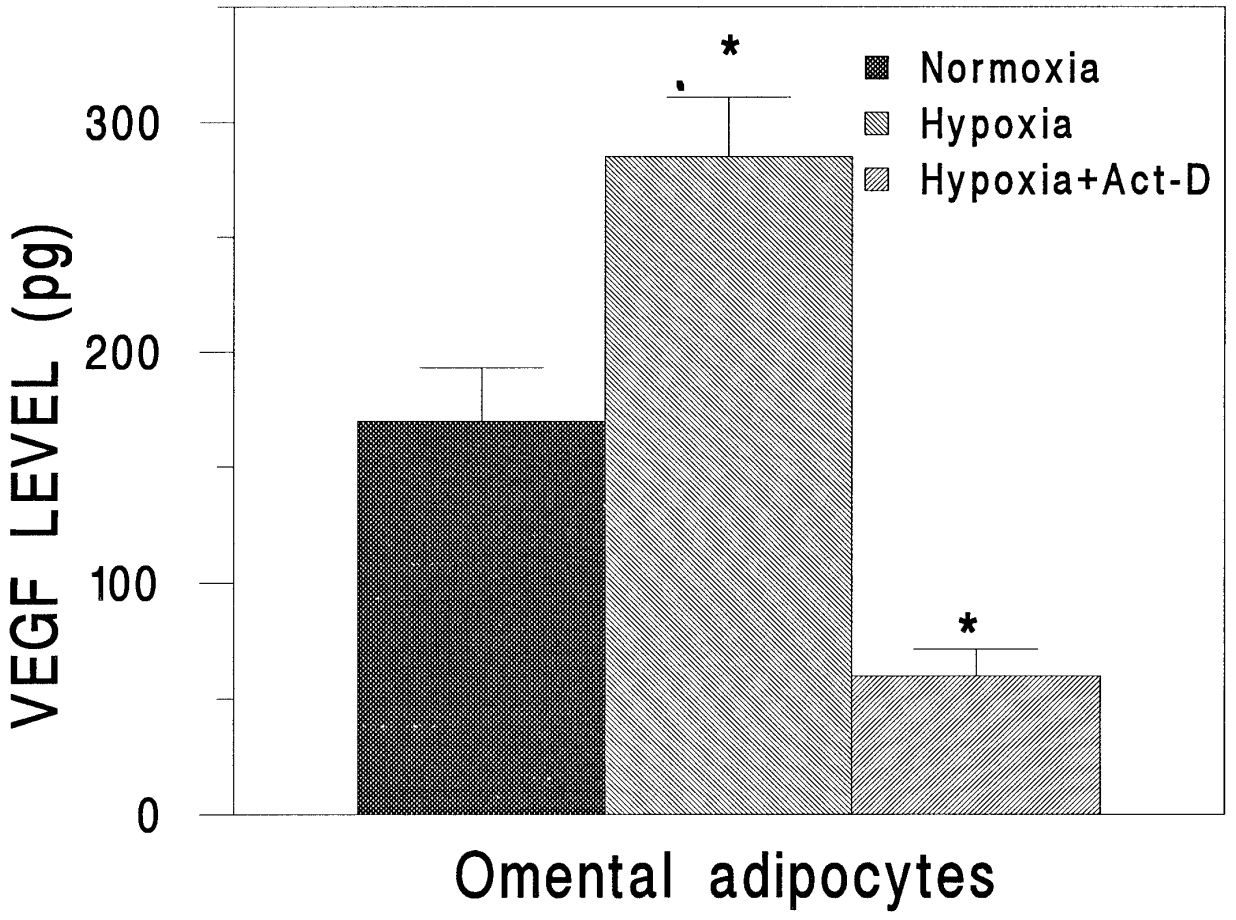

FIG. 6. Hypoxia induction of VEGF protein secretion by the omental adipocytes. Primary adipocytes were isolated from the omentum and cultured under normoxic or hypoxic conditions with or without actinomycin-D (Act-D) at 10 μg/ml. The conditioned media were collected 24 hr later and the VEGF protein in the media were quantified by EIA. Three wells were assayed for each specimen. The data represent the amount of VEGF protein produced by 1×10^4 omental adipocytes in 18 hr. *$P < 0.05$ vs the normoxic control.

20], may be explained by the abundant VEGF expression and the high levels of VEGF secretion by omentum noted in this report. The abundant VEGF protein expression demonstrated in adipose tissues in this report furthermore suggests the possibility that adipose tissue may play a prominent biological role in stimulating angiogenesis in several foci *in vivo*. Our observation that VEGF antibody significantly decreased the endothelial cell mitogenic activities of the heparin-binding proteins derived from omentum supports the hypothesis that VEGF may in fact be the major mitogen responsible for the endothelial mitogenic property of the omentum. The retention of residual mitogenic activity by the heparin-binding proteins derived from omentum after VEGF antibody neutralization nevertheless suggests that other heparin-binding growth factors such as FGF may also contribute to the endothelial mitogenic activity of the omentum to a much lesser degree.

The conditioned medium represents the actual secreted component of growth factors and the heparin-purified fraction is composed of the steady-state level of all heparin-binding growth factors associated with the cells. The more pronounced ability of VEGF antibody to almost totally abolish the endothelial mitogenic activity of the primary omental adipocyte-conditioned media, compared with its lesser effect on heparin purified extraction of omentum, furthermore suggests that VEGF may be the predominant *secreted* angiogenic growth factor produced by the omentum, and may be solely responsible for the clinical efficacy of this tissue in enhancing the revascularization of contiguous tissues. Evidence for the upregulation of other proteins under ischemic conditions, including FGF [21], nevertheless requires that the role of these other substances in inducing angiogenesis under appropriate conditions cannot be excluded.

More relevant to this discussion, however, the previously demonstrated mitogenic and angiogenic properties of VEGF [22] and the current demonstration of the ability of adipose tissue to abundantly express and secrete VEGF suggest that the biological role of VEGF production in adipose tissue may be as a means of paracrine regulation of angiogenesis, especially since adipose tissue is a major conduit for the vasculature in adulthood. In this regard, it should be noted that adipocytes have previously been demonstrated to have other significant endocrine and paracrine activities. The observation that adipose tissues are supplied by a com-

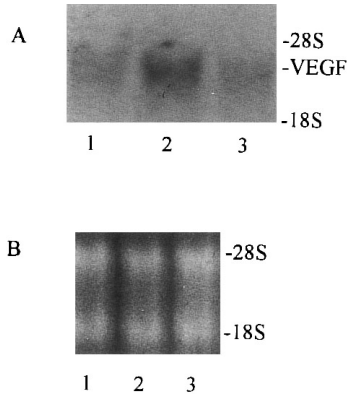

FIG. 7. Hypoxia-induced expression of VEGF-specific mRNA in omental adipocytes. Primary adipocytes were isolated from the omentum and cultured under normoxic or hypoxic conditions with or without actinomycin-D at 10 μg/ml. The total RNA from the cells was extracted and Northern blot was performed to analyze the expression of the VEGF-specific RNA as described under Materials and Methods. (A) Autoradiograph showing the hypoxia-induced expression of the VEGF specific mRNA. Lanes 1, normoxia-cultured omental adipocytes; 2, hypoxia-cultured omental adipocytes; 3, hypoxia-cultured omental adipocytes treated with actinomycin-D. The positions of the 28S, 18S, and VEGF-specific mRNA are indicated on the autoradiograph. (B) Ethidium bromide staining of the total RNA on the agarose gel. The total RNA from the cultured omental adipocytes was fractionated as described under Materials and Methods. Ethidium bromide staining demonstrated that equal amounts of the total RNA were loaded on the gel. The 28S and the 18S RNA are indicated. Lanes are assigned the same as in A.

paratively dense capillary network [23] and have been found to be innervated by sympathetic nerve fibers [24] further supports this consideration.

Our observation that hypoxia upregulates the expression of VEGF in cultured omental adipocytes suggests a regulatory pathway whereby hypoxia may initiate an increase in the VEGF response protein that in turn induces angiogenesis to relieve the inciting ischemic/hypoxic insult. Interestingly, the ability of omentum to induce an angiogenic response when surgically transplanted, while such a response is not seen in quiescent conditions, may be explained by two lines of evidence. First, some studies have demonstrated that injury alone induces an upregulation of VEGF expression [25] that is similar to that induced by hypoxia [26–28]. Second, FGF expression has been shown to be induced in normal tissues when placed in proximity to ischemic tissues [29], possibly through a cytokine regulatory mechanism similar to pathways described for the regulation of VEGF expression [30, 31].

In summary, our data demonstrate that abundant and differential expression of VEGF proteins exists in adipose tissues. The omentum contains and secretes the highest level of VEGF protein. Studies utilizing VEGF antibody furthermore suggest that VEGF is the major endothelial mitogen in the omentum and also in omental cell-conditioned medium. Finally, we have demonstrated that hypoxia can induce VEGF expression in primary omental adipocytes at the transcriptional level. These results suggest that modulation of the expression of VEGF in adipose tissue, by gene transfer or other techniques, may be a means of enhancing the clinical efficacy of angiogenic therapy, utilizing omentum or other tissues.

ACKNOWLEDGMENTS

We thank Drs. D. Hajjar and R. G. Crystal for providing many of the essential facilities during this study. The authors also thank Ms. L. Dinozo for editing the manuscript and secretarial assistance.

REFERENCES

1. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1:** 27, 1995.
2. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79:** 315, 1994.
3. Silverman, K. J., Lund, D. P., Zetter, B. R., Lainey, L. L., Shahood, J. A., Freiman, D. G., Folkman, J., and Berger, A. C. Angiogenic activity of adipose tissue. *Biochem. Biophys. Res. Commun.* **153:** 347, 1988.
4. Vineberg, A. M., Baichwal, K. S., and Myers, J. Treatment of acute myocardial infarction by epicardiectomy and free omental graft. *Surgery* **57:** 836, 1965.
5. Cooper, J. D., Pearson, F. G., Patterson, G. A., Todd, T. R. J., Ginsberg, R. J., Goldberg, M., and Demajo, W. A. P. Techniques of successful lung transplantation in humans. *J. Thoracic Cardiovasc. Surg.* **93:** 173, 1987.
6. Flier, J. S. The adipocyte: Storage depot or node on the energy information superhighway? *Cell* **80:** 15, 1995.
7. Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science* **259:** 89, 1993.
8. Goldsmith, H. S., Griffith, A. L., Kupferman, A., and Catsimprolas, N. Lipid angiogenic factor from omentum. *J. Am. Med. Assoc.* **252:** 2034, 1984.
9. Dobson, D. E., Kambe, A., Block, E., Dion, T., Lu, H., Castellot, J. J., and Spiegelman, B. M. 1-Butyryl-glycerol: A novel angiogenesis factor secreted by differentiating adipocytes. *Cell* **61:** 223, 1990.
10. Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.* **271**(2): 603, 1996.
11. Ferrara, N., Winer, J., and Burton, T. Aortic smooth muscle cells express and secrete vascular endothelial growth factor. *Growth Factors* **5:** 141, 1991.
12. Berse, B., Brown, L. F., Van De Water, L., Cvorak, H. F., and Senger, D. R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages and tumors. *Mol. Biol. Cell* **3:** 211, 1992.
13. Monacci, W. T., Merrill, M. J., and Oldfield, E. H. Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. *Am. J. Physiol.* **264**(*Cell Physiol.* 33): C995, 1993.
14. Ferrara, N., Leung, D. W., Cachianes, G., Winer, J., and Henzel, W. J. Purification and cloning of vascular endothelial growth factor secreted by pituitary folliculo-stellate cells. *Methods Enzymol.* **198:** 391, 1991.
15. Rodbell, M. J. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375, 1964.
16. Zhang, Q-X., Stanley, S. M., and Shah, G. V. Calcitonin inhibits prolactin gene transcription in rat pituitary cells. *Endocrine* **3:** 445, 1995.
17. Claffey, K. P., Wilkison, W. O., and Spiegelman, B. M. Vascular endothelial growth factor: Regulation by cell differentiation and

activated second messenger pathways. *J. Biol. Chem.* **267:** 16317, 1992.

18. Aversc, C. J. *Molecular Cell Biology.* Reading, MA: Addison–Wesley, 1986. Pp. 601–604.
19. Goldsmith, H. S., Duckett, S., and Chen, W. F. Prevention of cerebral infarction in the dog by intact omentum. *Am. J. Surg.* **130:** 317, 1975.
20. Goldsmith, H. S., Duckett, S., and Chen, W. F. Prevention of the cerebral infarction in the monkey by omental transposition to the brain. *Stroke* **9:** 224, 1978.
21. Rosengart, T. K., Duenas, M., Winkles, J., Krieger, K., and Isom, O. W. Ischemia is associated with increased expression of the angiogenic protein acidic fibroblast growth factor: Implications for "biologic" revascularization. *Surg. Forum* **45:** 392, 1994.
22. Connolly, D. T. Vascular permeability factor: A unique regulator of blood vessel function. *J. Cell. Biochem.* **47:** 219, 1991.
23. Roddie, I. C. Circulation to skin and adipose tissue. In J. T. Shepherd and F. M. Abboud (Eds.), *Handbook of Physiology,* Sect. 2, *The Cardiovascular System.* Bethesda, MD: Am. Physiol. Soc., 1983. Pp. 285–317.
24. Wasserman, F. The development of adipose tissue. In A. E. Renold and G. F. Cahill (Eds.), *Handbook of Physiology.* Washington, DC: Am. Physiol. Soc., 1965. Vol. 5, pp. 87–100.
25. Brown, L. F., Yeo, K. T., Berse, B., Yeo, T. K., Senger, D. R., Dvorak, H. F., and Van De Water, L. Expression of vascular endothelial growth factor by epidermal keratinocytes during wound healing. *J. Exp. Med.* **176:** 1375, 1992.
26. Shweiki, S., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359:** 843, 1992.
27. Ladoux, A., and Frelin, C. Hypoxia is a strong inducer of vascular endothelial growth factor in the heart. *Biochem. Biophys. Res. Commun.* **195:** 1005, 1991.
28. Goldberg, M. A., and Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J. Biol. Chem.* **269:** 4355, 1994.
29. Walgenbach, K. J., Gratas, C., Shestak, K. C., and Becker, D. Ischemia-induced expression of bFGF in normal skeletal muscle: A potential paracrine mechanism for mediating angiogenesis in ischemic skeletal muscle. *Nat. Med.* **1:** 453, 1995.
30. Dolecki, G. J., and Connolly, D. T. Effects of a variety of cytokines and inducing agents on vascular permeability factor mRNA levels in U937 cells. *Biochem. Biophys. Res. Commun.* **180:** 572, 1991.
31. Li, J., Perrella, M. A., Tsai, J-C., Yet, S-F., Hsiehe, C-M., Yoshizumi, M., Patterson, C., Eudege, W. O., Zhou, F., and Lee, M. E. Induction of vascular endothelial growth factor gene expression by interleukin-1B in rat aortic smooth muscle cells. *J. Biol. Chem.* **270**(1): 308, 1995.