VASCULAR ENDOTHELIAL GROWTH FACTOR RESTORES CORPOREAL SMOOTH MUSCLE FUNCTION IN VITRO

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ABSTRACT

Purpose: The therapeutic use of vasculogenic growth factors has been successfully demonstrated in models of organ ischemia. We determined whether vascular endothelial growth factor (VEGF) would reverse corporeal smooth muscle dysfunction in the hypercholesterolemic rabbit model of erectile dysfunction.

Materials and Methods: A total of 36 New Zealand White rabbits were fed a normal (12) or 1% cholesterol (24) diet and treated after 6 weeks with 0.9 mg. VEGF or vehicle. At 6 weeks 24 rabbits received a single intracavernous dose and 12 received a single intravenous bolus of either drug. Ten days after injection corporeal smooth muscle function was analyzed after relaxation to acetylcholine and sodium nitroprusside using isometric tension studies. Corporeal sections were assessed for smooth muscle content with f-actin staining and VEGF expression by immunohistochemical study and enzyme-linked immunosorbent assay.

Results: Endothelium dependent (acetylcholine) and nitric oxide mediated (sodium nitroprusside) smooth muscle relaxation were impaired in cholesterol fed animals ($p = 0.021$ and 0.003, respectively). Intracavernous VEGF treatment restored sodium nitroprusside mediated relaxation to normal ($p = 0.015$) and intravenous VEGF restored acetylcholine and sodium nitroprusside mediated relaxation ($p = 0.014$ and 0.018, respectively). Decreased smooth muscle content was noted in cholesterol fed animals versus normal diet controls ($p = 0.008$), which was not affected by VEGF treatment ($p = 0.450$). Corporeal endothelial cell content was increased after intracavernous but not intravenous VEGF treatment ($p = 0.001$ and 0.385, respectively). VEGF expression was augmented after treatment with recombinant VEGF ($p < 0.001$).

Conclusions: VEGF administration variably mitigated the impairment of corporeal smooth muscle relaxation in the hypercholesterolemic rabbit model of erectile dysfunction.

KEY WORDS: penis, penile erection, endothelial growth factors, hypercholesterolemia, rabbits

Penile erection is predominantly a vascular event and corporeal vasoactive function is affected by endothelium dependent and neurogenic mechanisms. Nitric oxide mediated trabecular smooth muscle relaxation is the final common pathway in normal penile erection.[1] Acetylcholine interacts with receptors on corporeal endothelial cells, resulting in the release of nitric oxide. Likewise nitric oxide is released from efferent nerves, which is thought to be the most important mechanism of normal penile erection. Direct measurement of the corporeal smooth muscle vasoactive response to acetylcholine or the nitric oxide donor sodium nitroprusside has been done in animal studies and clinical trials to assess erectile function.

Vascular endothelial growth factor (VEGF) is an endothelial cell specific mitogen in vitro and an angiogenic growth factor in vivo.[2–5] VEGF is produced by various cells, including vascular smooth muscle, endothelial and inflammatory cells, and has direct effects on vascular endothelial and smooth muscle cells through the activity of receptor tyrosine kinases.[6,7] VEGF has been shown to increase the ability of endothelial cells to produce nitric oxide, which may improve endothelial function.[2,4] When given experimentally, VEGF has been shown to significantly improve blood flow in vivo in chronic ischemic disorders, including ischemic heart and limb models.[2,3,8,9] It is thought that improved blood flow in these models is due to angiogenesis (new blood vessel development) and improved vasoactive function. Multicenter

trials are underway to assess the efficacy of VEGF therapy in patients with end stage coronary artery disease. Recently VEGF has been observed in human and rat corporeal tissue.[10] However, to our knowledge the therapeutic use of angiogenic growth factors in erectile dysfunction has not been previously explored.

Initially described in 1991 and further characterized at our laboratory, the hypercholesterolemic rabbit model of erectile dysfunction is a reproducible method for studying corporeal smooth muscle function and dysfunction in vitro.[11–14] This model produces endothelium dependent and direct nitric oxide mediated corporeal smooth muscle dysfunction after 8 weeks of a high cholesterol diet, as measured by isometric tension studies. Although strictly an in vitro assay, they have been studied as animal models of erectile dysfunction. This and similar rabbit models have been evaluated by measurement, including intracavernous blood flow, intracavernous pressure decay and cavernous expandability, and hypercholesterolemic rabbits have been shown to have deficits compared with normal animals.[14,15] We assessed the effects of VEGF as a potential treatment for erectile dysfunction by analyzing its effects on corporeal smooth muscle dysfunction produced by the hypercholesterolemic rabbit model.

METHODS

In a protocol approved by the Institutional Animal Care and Use Committee at Duke University Medical Center 36 New Zealand White rabbits were fed a normal rabbit (12) or

1% cholesterol (24) diet (Harland Teklab, Madison, Wisconsin) for 7.5 weeks. A total of 24 rabbits (half normal and half cholesterol diet) received an intracavernous injection of 0.9 mg. recombinant VEGF-165 or an equivalent amount of vehicle (Genentec, South San Francisco, California) at week 6. A total of 12 cholesterol fed rabbits received a single intravenous injection of 0.9 mg. VEGF or vehicle at week 6 (table 1). Ten days after injection rabbits were sacrificed and penectomy was done with meticulous dissection of the corpora cavernosa from the tunica albuginea. Total serum cholesterol was determined before the initiation of the experimental diet and immediately before the procedure.

As previously described,[13] for isometric tension studies 2 corporeal strips from each animal were suspended in 5 ml. capacity organ baths containing Krebs physiological salt solution (122 mmol./l. NaCl, 4.7 mmol./l. KCl, 1.2 mmol./l. $MgCl_2$, 2.5 mmol./l. $CaCl_2$, 15.4 mmol./l. $NaHCO_3$, 1.2 mmol./l. KH_2PO_4 and 5.5 mmol./l. glucose) maintained at 37C and oxygenated with 95% O_2 and 5% CO_2. After equilibration at 0.5 gm. optimal preload tension was determined by contracting strips with 60 mmol. KCl Krebs solution (60 mmol./l. NaCl, 1.2 mmol./l. $MgCl_2$, 2.5 mmol./l. $CaCl_2$, 15.4 mmol./l. $NaHCO_3$, 1.2 mmol./l. KH_2PO_4 and 5.5 mmol./l. glucose) at incrementally increasing levels of pre-load until a further increase in tension failed to generate an increase in active tension (total tension minus resting tension) of at least 10%. All subsequent testing was then performed at the optimal resting tension for each strip. Strips were sub-maximally pre-contracted with 10^{-5} M. norepinephrine and after a contractile plateau was reached 10^{-8} to 10^{-3} M. acetylcholine or 10^{-8} to 10^{-4} M. sodium nitroprusside was added cumulatively in logarithmic increments. Endothelial dependent relaxation was assessed using acetylcholine, while direct nitric oxide mediated corporeal smooth muscle dysfunction relaxation was assessed by sodium nitroprusside. Electrical field stimulation was not performed. Relaxation in response to each dose of acetylcholine or sodium nitroprusside is expressed as a percent of the active tension generated by the 10^{-5} M. dose of norepinephrine. These values were plotted against the negative logarithm of the agonist dose to produce relaxation dose response curves. Logistic regression analysis with logit transformation was done on the cumulative dose response curves of each treatment group to determine the ED25, ED50 and ED75 of each agent.[13, 16] Treatment groups were compared at the ED50 of acetylcholine and sodium nitroprusside, and at maximal relaxation to each agent.

To define antibodies better and determine which VEGF antibody (antihuman or rabbit) immunohistochemical studies were done. Corporeal sections were cryopreserved in 30% sucrose, snap frozen and sectioned at 5 μm. After acetone fixation for 10 minutes phosphate buffered saline wash and incubation with blocking solution (10% horse serum in phosphate buffered saline), primary antibody was applied. The HHF35 monoclonal mouse antibody to f-actin incubated for 30 minutes was used for actin immuno-expression and smooth muscle quantification (Dako, Carpinteria, California). A monoclonal mouse antibody to CD-31 incubated overnight was used for CD-31 immuno-expression and endothelial quantification (Biogenics, San Ramon, California). A monoclonal horse VEGF antibody incubated for 1 hour was used for VEGF immuno-expression (Sigma Chemical Co., St. Louis, Missouri). Antigens were developed with ABC reagent and the alkaline phosphatase substrate kit (Vector Labora-

tories, Burlingame, California) with hematoxylin counterstaining, rendering antigen expressing areas red. Ten randomly selected ×40 fields per animal in each treatment group were analyzed using an image analysis system and overall smooth muscle (actin) or endothelial (CD-31) area was quantified using National Institutes of Health image software. Likewise 10 randomly selected ×40 VEGF stained fields per animal in each treatment group were assessed and areas of VEGF expression were counted by a single observer blinded to treatment group. The smooth muscle, endothelial and VEGF contents measured in normal diet, vehicle treated rabbits were assigned a value of 100%, presented as the mean plus or minus standard error of mean, and other treatment groups were assessed relative to these values. Intracavernous and intravenous groups were evaluated using the same protocol but at different times with different antibody lots and, thus, direct comparison of staining patterns by these 2 routes may be biased.

We performed enzyme-linked immunosorbent assay (ELISA) of VEGF expression. Frozen corporeal tissues were sonicated in 50 mM. tris radio-immunoprecipitation assay buffer for 1 minute for protein isolation. Protein concentrations were determined using the Bradford protein assay and 60 μg. of protein per sample were boiled for 5 minutes before analysis using a Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, Minnesota). Samples were quantified with a Molecular Devices Kinetic microplate reader (Sunnyvale, Calif) using Apple SoftMax software (Apple Computers, Sunnyvale, California). An ELISA was performed for each animal in each treatment group.

Data are expressed as the mean plus or minus standard error of mean (SEM). The response of strips from each treatment group to acetylcholine or sodium nitroprusside was compared using the independent samples t test after samples were examined for normality and no significant deviations were observed. Statistical significance was determined at p = 0.05.

RESULTS

Serum cholesterol increased from a mean of 63.1 ± 3.7 to 1501.0 ± 47.1 mg./dl. after 7½ weeks on the 1% cholesterol diet (p <0.001). There was no difference in the final mean cholesterol level in the VEGF and vehicle treated groups (1,565.2 ± 76.9 and 1,499.3 ± 98.9 mg./dl., respectively, p = 0.605). The final mean serum cholesterol level in control animals was 60.1 ± 1.4 mg./dl.

On isometric tension studies the ED50 ($-$log [M]) to acetylcholine mediated, endothelium dependent relaxation was decreased in cholesterol fed animals compared with in normal controls (table 2). The ED50 and maximal relaxation to direct nitric oxide mediated (sodium nitroprusside) relaxation were decreased in cholesterol fed compared with normal diet animals (table 3).

Endothelium dependent smooth muscle relaxation was not affected by intracavernous VEGF treatment (fig. 1, A and table 2). In cholesterol fed animals treated with intracavernous VEGF or vehicle there was no significant difference in the ED50 to acetylcholine relaxation or the maximal relaxation to acetylcholine. However, nitric oxide mediated, direct smooth muscle relaxation was significantly improved in cholesterol fed, intracavernous VEGF treated rabbits (fig. 1, B and table 3). While mean sodium nitroprusside relaxation was not significantly different at the ED50, the dose response curve was shifted to the left in VEGF treated rabbits and this difference may be significant at the ED75 (5.75% ± 0.16% and 5.23% ± 0.18%, p = 0.046). Moreover, maximal relaxation to sodium nitroprusside was significantly augmented in intracavernous VEGF treated animals compared with vehicle controls. Endothelium dependent smooth muscle relaxation was augmented by intravenous VEGF treatment with a

TABLE 1. *Treatment groups according to diet and therapy*

Animal Diet	Intracavernous Vehicle (No. subjects)	Intracavernous VEGF (No. subjects)	Intravenous Vehicle (No. subjects)	Intravenous VEGF (No. subjects)
1% Cholesterol	6	6	6	6
Regular	6	6	0	0

TABLE 2. *Acetylcholine isometric tension studies*

	Mean 50% Effective Dose ±SEM (−log [M])	p Value	Mean % Maximal Relaxation ±SEM	p Value
Cholesterol, intracavernous vehicle	5.29 ± 0.43	0.021	87.8 ± 9.2	0.613
Normal diet, intracavernous vehicle	6.50 ± 0.19		92.8 ± 2.5	
Cholesterol, intracavernous VEGF	4.94 ± 0.25	0.384	78.6 ± 6.2	0.667
Cholesterol, intracavernous vehicle	5.29 ± 0.43		87.8 ± 9.2	
Cholesterol, intravenous VEGF	6.33 ± 0.40	0.004	96.1 ± 8.9	0.014
Cholesterol, intravenous vehicle	4.83 ± 0.25		71.6 ± 4.0	

TABLE 3. *Sodium nitroprusside isometric tension studies*

|	Mean 50% Effective Dose ±SEM (−log [M])	p Value	Mean % Maximal Relaxation ±SEM	p Value
Cholesterol, intracavernous vehicle	6.00 ± 0.18	0.003	95.2 ± 3.5	<0.001
Normal diet, intracavernous vehicle	6.82 ± 0.16		125.1 ± 6.8	
Cholesterol, intracavernous VEGF	6.36 ± 0.16	0.148	113.9 ± 6.3	0.015
Cholesterol, intracavernous vehicle	6.00 ± 0.18		95.2 ± 3.5	
Cholesterol, intravenous VEGF	6.93 ± 0.25	0.001	116.6 ± 7.8	0.018
Cholesterol, intravenous vehicle	5.70 ± 0.13		95.6 ± 3.6	
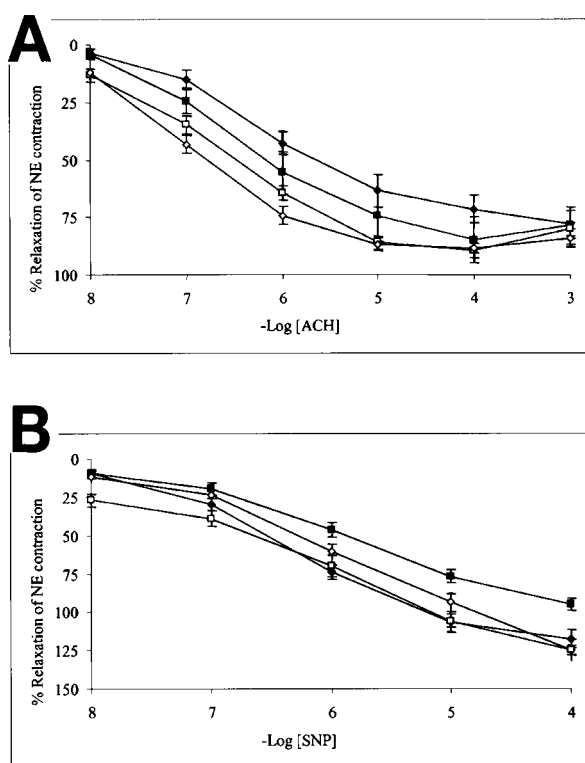
FIG. 1. Isometric tension studies after intracavernous VEGF therapy. *A*, endothelium dependent smooth muscle relaxation was not affected by VEGF treatment. Black diamonds represent cholesterol fed, VEGF treated animals. Black squares represent cholesterol fed, vehicle treated animals. Open diamonds represent normal diet, VEGF treated animals. Open squares represent normal diet, vehicle treated animals. *NE*, norepinephrine. *ACH*, acetylcholine. *B*, nitric oxide mediated, direct smooth muscle relaxation was improved in cholesterol fed, VEGF treated rabbits at ED75 (p = 0.046) and at maximal relaxation (p = 0.015). *SNP*, sodium nitroprusside.
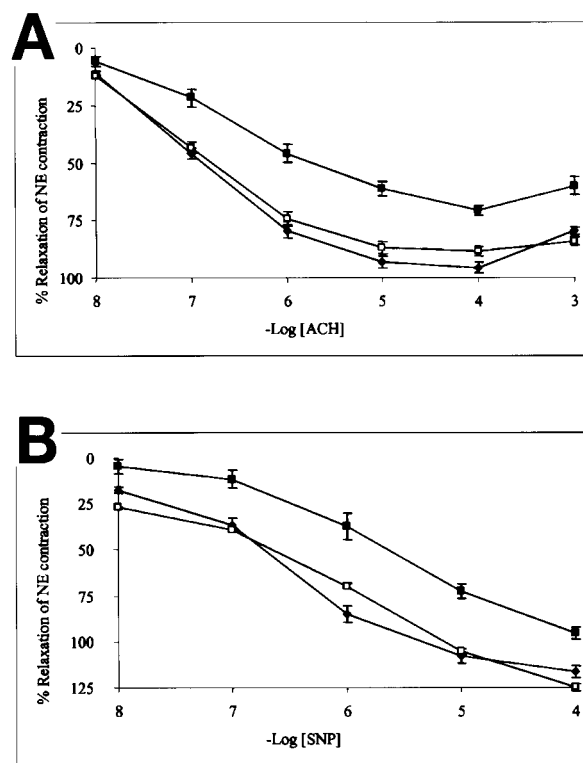

FIG. 2. Isometric tension studies after intravenous VEGF therapy. *A*, endothelium dependent smooth muscle relaxation was potentiated by VEGF treatment at ED50 (p = 0.004) and at maximal relaxation (p = 0.014). Black diamonds represent cholesterol fed, VEGF treated animals. Black squares represent cholesterol fed, vehicle treated animals. Open diamonds represent normal diet, VEGF treated animals. Open squares represent normal diet, intracavernous vehicle treated animals. *NE*, XXX. *ACH*, acetylcholine. *B*, nitric oxide mediated, direct smooth muscle relaxation was improved in cholesterol fed, VEGF treated rabbits at ED50 (p = 0.001) and at maximal relaxation (p = 0.018). *SNP*, sodium nitroprusside.

significant difference in the ED50 to acetylcholine relaxation and maximal relaxation to acetylcholine (fig. 2, *A* and table 2). Likewise sodium nitroprusside mediated, direct smooth muscle relaxation was significantly improved in cholesterol fed, intravenous VEGF treated rabbits at the ED50 and maximal relaxation (fig. 2, *B* and table 3).

As measured by CD-31 staining, mean endothelial cell content was significantly augmented in normal diet animals after intracavernous VEGF versus vehicle administration (136% ± 6% versus 100% ± 5%, p = 0.001). In cholesterol fed animals mean endothelial cell content was increased after

intracavernous VEGF versus vehicle administration (114% ± 6% versus 81% ± 6%, p = 0.006). However, after intravenous VEGF versus vehicle was given this difference was not evident (83% ± 4% and 87% ± 5%, respectively, p = 0.385, fig. 3)

Cholesterol diet, vehicle treated animals had significantly decreased mean overall trabecular smooth muscle content by actin staining compared with normal diet controls (56% ± 3% versus 100% ± 3%, p = <0.001). Mean smooth muscle content did not differ in cholesterol fed rabbits whether given
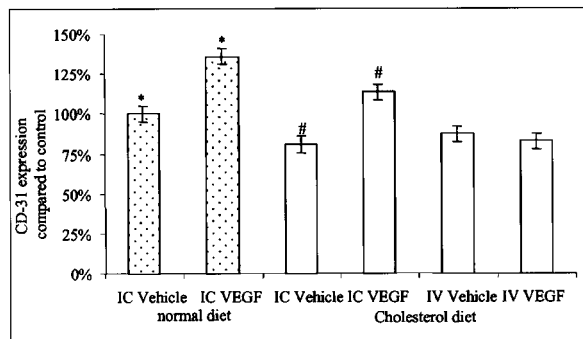
FIG. 3. Endothelial cell content in various treatment groups, as measured by CD-31 immunostaining. Asterisk represents statistically significant difference in 2 treatment group on normal diet. Pound sign represents statistically significant difference in 2 treatment groups fed cholesterol. *IC*, intracavernous. *IV*, intravenous.

intracavernous VEGF or vehicle (63% ± 2% and 56% ± 3%, respectively, p = 0.087). Likewise mean actin staining was not significantly different in the intravenous VEGF and intravenous vehicle groups (41% ± 2% and 46% ± 3%, respectively, p = 0.102, fig. 4)

Cholesterol diet, vehicle treated animals had significantly decreased mean VEGF immuno-expression than normal diet controls (44% ± 6% versus 100% ± 4%, p <0.001). Of the normal diet rabbits VEGF treated animals had higher mean VEGF expression than vehicle treated animals (151% ± 4% versus 100% ± 4%, p <0.001). In cholesterol fed animals intracavernous VEGF treatment significantly augmented mean VEGF expression compared with vehicle treated controls (79% ± 6% versus 44% ± 6%, p < 0.001, fig. 5).

On VEGF ELISA optical density minus background in cholesterol fed animals treated with VEGF was greater than that in vehicle treated animals, although this value did not reach statistical significance (0.168 versus 0.094, p = 0.086). Likewise optical density was increased after intravenous VEGF administration compared with after vehicle (0.128 versus 0.91, p = 0.519).

DISCUSSION

Vascular endothelial growth factor is under trial in animal models and patients with coronary or peripheral vascular ischemic disorders but its use in erectile dysfunction has not previously been described.[2,3,8,9] However, recently VEGF has been demonstrated in human and rat corporeal tissue.[10] VEGF is an endothelial cell specific mitogen in vitro and an angiogenic growth factor in vivo.[2–5] It is thought that improved blood flow with VEGF treatment is due to angiogenesis and improved vasoactive function.
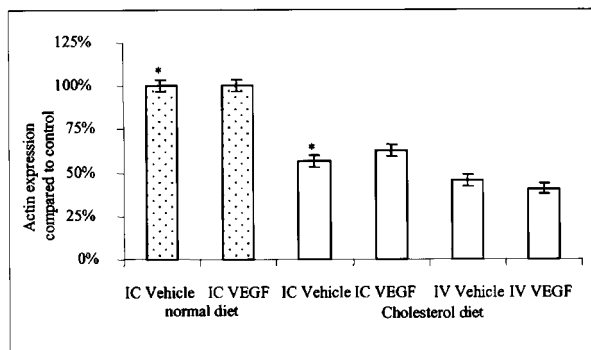

FIG. 4. Smooth muscle content in various treatment groups, as measured by actin immunostaining. Asterisk represents statistically significant difference in 2 treatment groups. *IC*, intracavernous. *IV*, intravenous.

In men every mmol./l. increase in total cholesterol results in a 1.32-fold increase in the risk of erectile dysfunction[17] and hypercholesterolemia is a common component of many animal models of erectile dysfunction. Although the hypercholesterolemic rabbit model of erectile dysfunction is associated with superphysiological serum cholesterol, it has been established as a reproducible method for studying corporeal smooth muscle function and dysfunction in vitro.[11–14] Previous reports have shown morphological changes in erectile tissue subjected to hypercholesterolemia, including focal areas of endothelial cell disruption, vacuolated endothelial cells and increased lipid laden vesicles within smooth muscle cells.[13] Others have reported that a cholesterol diet causes the percent of rabbit corporeal smooth muscle cells to significantly decrease from 45.4% to 39.2%,[14] similar to the decrease in men with veno-occlusive erectile dysfunction.

VEGF has been shown to increase the ability of endothelial cells to produce nitric oxide, which may improve endothelial function.[2,4] Sinusoidal endothelium has an important role in regulating cavernous smooth muscle tone and acetylcholine induced smooth muscle relaxation relies on it. Although in our experiments intracavernous VEGF did not appear to have an effect, we observed improved endothelium dependent corporeal smooth muscle relaxation in rabbits fed a cholesterol diet and treated with an intravenous bolus of VEGF versus vehicle (figs. 1a, 2a). Experience with VEGF in other systems implies that various delivery routes may produce various effects,[18] which may explain the disparity in our experiments. The demonstrated corporeal effects of intravenous VEGF may be a result of changes at the level of the systemic vasculature, perhaps allowing improved blood flow to the penis. Because the systemic vasculature was not evaluated in this study, this result is speculative and should be assessed in future studies. Previous studies in hypercholesterolemic rabbits have shown iliac arteries with a thickened intima and moderate fibrosis but normal lumen.[19] Alternatively the acetylcholine mediated response may have been present in the intracavernous cohort but not evident due to technical reasons associated with the fragility of rabbit vascular endothelium and assay limits. Further studies are needed to clarify this possibility. To determine whether the observed improved response was associated with an increased number of endothelial cells CD-31 immunohistochemical expression was assessed. CD-31 is an endothelium specific cell marker that has been conventionally used to assess angiogenesis. We noted increased CD-31 staining after intracavernous VEGF treatment, which may correspond to the increased endothelial cell population (fig. 3). However, this increase was not apparent after intravenous administration.

Sodium nitroprusside is metabolized by smooth muscles into nitric oxide, acts as a nitric oxide donor and directly assesses smooth muscle function.[20] Our results imply that VEGF, in addition to an endothelial dependent response, results in an improved direct nitric oxide response of corporeal smooth muscle (fig. 1 and table 3). Wang and Keiser have described direct effects of VEGF on vascular smooth muscle cells, including up-regulation of matrix metalloproteinase production and augmented smooth muscle cell migration, mediated through VEGF stimulated phosphorylation of the smooth muscle cell flk-1 receptor.[7] They demonstrate matrix metalloproteinase mediated stimulation of vascular smooth muscle cell migration by in vitro invasion assay, implying that this effect may be partially responsible for the migration of perivascular mesenchymal cells and smooth muscle cells to angiogenesis sites. To determine whether improved nitric oxide mediated smooth muscle relaxation after VEGF therapy was secondary to augmented smooth muscle content rather than to enhanced smooth muscle function we quantified smooth muscle content. Consistent with
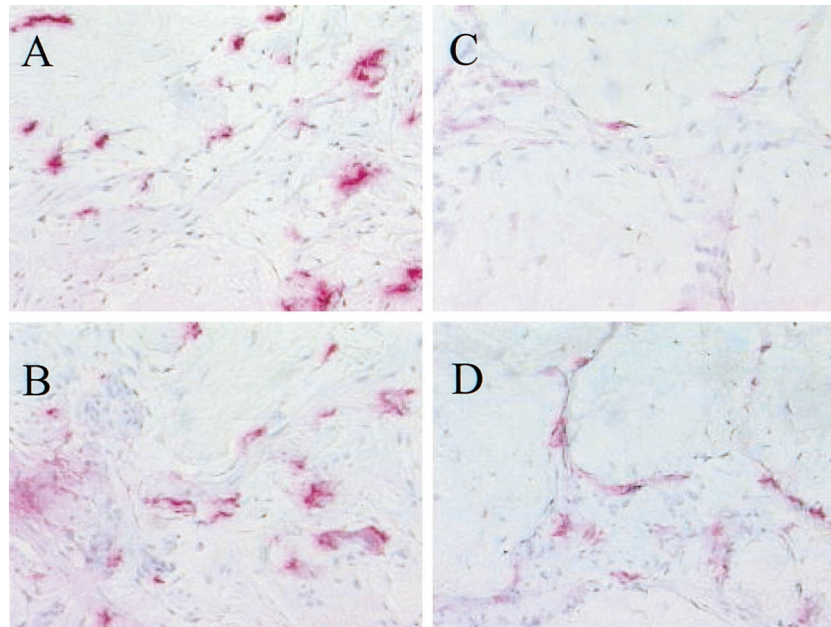
Fig. 5. VEGF immuno-expression. *A*, normal diet, intracavernous vehicle treated rabbits. *B*, normal diet, intracavernous VEGF treated rabbits. *C*, cholesterol diet, intracavernous vehicle treated rabbits. *D*, cholesterol diet, intracavernous VEGF treated rabbits.

previous reports, trabecular smooth muscle content was decreased in cholesterol fed animals. However, we noted no statistical difference in smooth muscle content in the VEGF or vehicle treated, cholesterol fed rabbits (fig. 4).

We observed decreased immunohistochemical expression of VEGF in animals subjected to experimental hypercholesterolemia. Furthermore, we noted that VEGF immuno-expression was augmented in normal diet and cholesterol fed rabbits after a single bolus of VEGF. This effect was further demonstrated by ELISA, although small sample size may have limited the statistical significance of this assay. The observed increase in VEGF expression may represent enhanced endogenous production of VEGF but further studies are needed to ensure that the immunohistochemical increase is not a result of detecting residual exogenous recombinant VEGF.

The finding that VEGF administration durably restored the corporeal smooth muscle vasoactive response in the hypercholesterolemic rabbit model may be clinically important and further study is indicated to determine whether it may have a similar effect in men with impotence. The therapeutic use of VEGF may provide a means of restoring trabecular smooth muscle function to a level adequate for effective venous occlusion when given alone or with pharmacological assistance. Moreover, VEGF therapy may become an important adjunct to novel tissue engineering approaches to erectile dysfunction.[21]

CONCLUSIONS

We have demonstrated that exogenous VEGF may improve vasoactive corporeal smooth muscle dysfunction in rabbits exposed to superphysiological levels of cholesterol. Further studies are needed to elucidate the mechanism of the functional improvement demonstrated. Studies in other models, and with other dosing routes and regimens are indicated to examine this therapy in the absence of superphysiological experimental hypercholesterolemia.

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