Aging is associated with an impaired coronary microvascular response to vascular endothelial growth factor in patients

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Objective: If growth factor and cell-based therapy are to become therapeutic strategies, a better understanding of their physiologic effects in various patient populations will need to be gained. In this study, we examined age-dependent differences in vascular endothelial growth factor signaling before and after cardioplegia/cardiopulmonary bypass.

Methods: Atrial tissue and peripheral blood of patients undergoing surgery were examined before and after cardioplegia/cardiopulmonary bypass. Patients were divided into younger (age < 70) and older (age ≥ 70) groups. Coronary microvascular responses, expression of vascular endothelial growth factor and its downstream signaling molecules, and the number of CD34⁺ progenitor cells before and after cardioplegia/cardiopulmonary bypass were compared between groups.

Results: Advanced age was associated with impaired basal coronary microvascular response to vascular endothelial growth factor ($-13\% \pm 5\%$ at 10^{-10} mol/L vascular endothelial growth factor; $P = .04$), whereas basal relaxation response to substance P and sodium nitroprusside were similar between groups. After cardioplegia/cardiopulmonary bypass, the microvascular response to vascular endothelial growth factor significantly worsened in both groups (both $P \leq .05$), and response to substance P ($P = .05$) was significantly impaired only in older patients. Vascular endothelial growth factor expression increased after cardioplegia/cardiopulmonary bypass in older ($P = .01$ vs before cardioplegia/cardiopulmonary bypass), but not younger ($P = .20$) patients. Expression of other signaling molecules was unaffected by age or surgery. Circulating CD34⁺ cells increased after cardioplegia/cardiopulmonary bypass in all patients but to a greater extent in younger patients ($P = .01$).

Conclusions: The coronary microvascular response to vascular endothelial growth factor is impaired in older patients. Combined with reduced progenitor cell mobilization, these results suggest new mechanisms for reduced angiogenic response in older patients.

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This work was supported by National Institutes of Health grant (K12), Junior Faculty Award on Aging, Harvard Medical School (Jian Li), and National Institutes of Health grants R01 HL46716 (Frank W. Sellke) and R01 HL69024 (Frank W. Sellke).

Read at the Eighty-sixth Annual Meeting of The American Association for Thoracic Surgery, Philadelphia, Pa, April 29-May 3, 2006.

Received for publication April 26, 2006; revisions received June 26, 2006; accepted for publication Aug 3, 2006.

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J Thorac Cardiovasc Surg 2006;132:1348-55
0022-5223/\$32.00

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doi:10.1016/j.jtcvs.2006.08.043

Protein and gene therapy using growth factors and cell therapy using bone marrow-derived stem cells are promising therapeutic strategies to induce angiogenesis for ischemic heart disease in combination with conventional revascularization techniques.^{1,2} Vascular endothelial growth factor (VEGF) has been shown to be a potent angiogenic agent in swine models of chronic myocardial ischemia.³⁻⁵ However, clinical trials to date have not demonstrated the tremendous effects of angiogenesis shown in animal studies.^{6,7}

Angiogenesis is induced in response to tissue injury. In this context, vasodilation and mobilization of stem cell progenitors are important early events in the angiogenic process. VEGF plays an important role in coronary microvascular vasodilatation mediated through nitric oxide (NO).⁸ We⁹ have previously shown that

Abbreviations and Acronyms

CP	= cardioplegia
CPB	= cardiopulmonary bypass
eNOS	= endothelial nitric oxide synthase
G-CSF	= granulocyte-colony stimulating factor
NO	= nitric oxide
NOS	= nitric oxide synthase
PB	= peripheral blood
SNP	= sodium nitroprusside
VEGF	= vascular endothelial growth factor
VEGFR2	= vascular endothelial growth factor receptor 2

NO bioavailability in coronary microvessels is decreased after cardioplegia and cardiopulmonary bypass (CP/CPB). Furthermore, it has been shown that stem cell progenitors are mobilized from bone marrow, resulting in elevation in their circulating numbers after CP/CPB.^{10,11} These studies reinforce the idea that exposure to CP/CPB can modulate important early events in angiogenesis with potential implications for growth factor-based angiogenic therapy administered at the time of surgical revascularization.

Advanced age remains an important risk factor for coronary disease and has also been associated with reduced NO availability and endothelial dysfunction.^{12,13} Heiss and associates¹⁴ have demonstrated that flow-mediated dilatation of the brachial artery in response to the NO-dependent vasodilator, acetylcholine, is impaired in older patients. Furthermore, the number of circulating endothelial progenitor cells is also reduced in the elderly.

The purposes of this study were (1) to evaluate age-dependent differences in human coronary microvascular response and to examine the expression of VEGF and its downstream mediators before and after CP/CPB and (2) to investigate the effect of age on the mobilization of circulating CD34⁺ stem progenitors after CP/CPB.

Materials and Methods**Human Tissue Samples**

This study was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center, Harvard Medical School. Informed consent was obtained from patients enrolled in the study as required by the Institutional Review Board. Patients undergoing coronary and/or valvular surgery with CPB were enrolled. Samples of atrial tissue were harvested from the right atrial appendage before and after CP/CPB by a double purse-string technique as previously described.¹⁵ The first specimen was taken at the time of atrial cannulation. The second specimen was collected by securing the second purse-string suture below the cannulation site after weaning from CPB and at the time of decannulation. Tissue samples were immediately placed in cold (5°C–10°C) Krebs buffer solution for microvessel studies (n = 16) or frozen in liquid nitrogen and stored at –80°C in preparation for Western blotting (n = 10). Because the median value of these 16 samples was 69.5 years old, we divided the samples into older (age ≥

70) and younger (age < 70) age groups. Peripheral blood samples were taken before skin incision (pre-CP/CPB) and 4 hours after surgery (n = 16). Blood was collected for flow cytometry analysis and measurement of serum levels of granulocyte-colony stimulating factor (G-CSF).

Coronary microvessel studies. Microvessels (50–150 μ m internal diameters) were dissected from atrial tissue with a dissecting microscope (Olympus Optical, Tokyo, Japan) at original magnifications of 10 $\times$ to 60 $\times$. Microvessels were placed in a microvessel chamber (University of Iowa Medical Instrumentation, Iowa City, Iowa), cannulated with dual glass micropipettes, and secured with 10-0 nylon monofilament suture (Ethicon, Inc, Somerville, NJ). Krebs buffer solution, warmed to 37°C, was continuously circulated through the microvessel chamber. The microvessels were pressurized to 40 mm Hg in a no-flow state with a burette manometer filled with Krebs buffer solution. With an inverted microscope (at original magnification 40 $\times$ to 200 $\times$; Olympus Optical, Tokyo, Japan) connected to a video camera, the vessel image was projected onto a television monitor. An electronic dimension analyzer (Living System Instrumentation, Burlington, Vt) was used to measure internal lumen diameter, and measurements were recorded. After precontraction of microvessels by 30% to 50% of the baseline diameter with the thromboxane A₂ analog U46619 (0.1–1.0 μ mol/L), microvessel responses to VEGF₁₆₅ (10^{–15}–10^{–10} nmol/L) were examined. In addition, the response to sodium nitroprusside (SNP) (10^{–9}–10^{–4} mol/L), an endothelium-independent cyclic guanosine monophosphate-mediated vasodilator, and substance P (10^{–14}–10^{–7} M), an endothelium-dependent vasodilator, was examined. Selected experiments were performed on vessels in the presence of NO synthase (NOS) inhibitor, nitro-L-arginine (10^{–4} mol/L). Human VEGF₁₆₅ was purchased from R&D Systems (Minneapolis, Minn). SNP, nitro-L-arginine, U46619, and substance P were purchased from Sigma Chemical Company (St Louis, Mo).

Western blot analysis. Whole-cell lysates were isolated from the atrial tissue taken before and after CP/CPB (n = 10) with a RIPA buffer (Boston Bioproducts, Worcester, Mass) and centrifuged at 12,000g for 10 minutes at 4°C to separate soluble from insoluble fractions. The supernatant protein concentration was measured spectrophotometrically at a 595-nm wave length. Total protein was fractionated by 4% to 20% gradient, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Invitrogen, San Diego, Calif), and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass). The membranes were incubated overnight at 4°C using specific antibodies as follows, anti-VEGF antibody diluted to 1:250, anti-VEGF receptor 2 (VEGFR2) antibody diluted to 1:200, anti-Akt antibody diluted to 1:1000 (Santa Cruz Biotechnology, Santa Cruz, Calif), phospho-Akt (Ser473) antibody diluted to 1:1000 (Cell Signaling Technology, Inc, Danvers, Mass), and anti-endothelial NOS (eNOS) antibody diluted to 1:2500 (BD Bioscience, San Jose, Calif). Then, the membranes incubated for 1 hour in diluted appropriate secondary antibody (Jackson Immuno-lab, West Grove, Pa). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Specific protein densities were measured by densitometric quantification of autoradiograph films using NIH image J 1.33 (National Institutes of Health, Bethesda, Md). Equal loading of various samples was confirmed by Ponceau S staining.

Flow cytometry analysis. The sample of whole blood (100 μ L) was stained with fluorescein isothiocyanate-conjugated anti-CD34 (BD Bioscience, San Jose, Calif) monoclonal antibodies for 30 minutes at 4°C ($n = 16$). Red blood cells and platelets were subsequently lysed for 15 minutes by an erythrocyte lysing buffer (Quiagen, Valencia, Calif), and the sample was centrifuged, washed twice, resuspended in phosphate-buffered saline solution, and analyzed by use of a FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Isotype-matched fluorescein isothiocyanate-conjugated IgG1 antibodies (eBioscience) were used as controls. The number of CD34⁺ cells was obtained as the absolute number in a total of 50,000 leukocytes. Leukocytes were stained by 0.15% Trypan blue and counted using the lysed sample for flow cytometry (FACS) without antibody. The number of CD34⁺ cells in 100 μ L of peripheral blood (PB) was calculated as follows:

Total cells = Leukocytes (cells/ μ L of PB) $\times$ 100 $\times$ (absolute number of CD34⁺ cells)/50,000

Enzyme-linked Immunosorbent Assay for G-CSF in Human Plasma

Blood was obtained from the patients before CPB and after CPB ($n = 16$). Serum samples were obtained after centrifugation. Quantitative immunoassays of G-CSF were performed by a G-CSF enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer's instructions.

Data Analysis

All results are expressed as mean $\pm$ SEM. Comparisons between age, VEGF concentration, and microvessel response were analyzed by repeated-measures analysis of variance. Western blots were analyzed after digitalization of x-ray films using a flat-bed scanner (ScanJet 4c; Hewlett Packard, Palo Alto, Calif) and NIH Image J 1.33 software (National Institutes of Health). Comparisons of Pre-CP/CPB samples between younger and older groups were analyzed by unpaired 2-tailed t test and comparisons between samples before and after CP/CPB were analyzed by paired 2-tailed t test. Statistical analyses were conducted with JMP 5.0 (SAS Institute Inc, Cary, NC) and Graph Pad Prism 4 (Graph Pad Software Inc, San Diego, Calif).

Results

Patient characteristics are depicted in Table 1.

Effects of Age on the Basal Coronary Microvessel Response

The coronary microvascular relaxation response to VEGF was diminished in older patients ($P = 0.04$). The responses to substance P ($P = .68$) and SNP ($P = .59$) were similar between the groups (Figure 1). After treatment of vessels with 10^{-4} mol/L of the NOS inhibitor nitro-L-arginine, the microvascular responses to VEGF (10^{-11} mol/L; $37.3\% \pm 16.5\%$ vs $14.9\% \pm 8.4\%$; $P = .02$) and substance P (10^{-6} mol/L; $48.1\% \pm 12.2\%$ vs $10.0\% \pm 6.5\%$; $P < .01$) were significantly blunted. These results suggest that the responses to VEGF and substance P are, in large part, mediated by NO release and that under basal conditions, advanced age is associated with impaired VEGF signaling in the coronary microcirculation.

Myocardial Expression of VEGF and Related Mediators

To further explore age-related differences in coronary microvascular response to VEGF, we evaluated the expression of VEGF and its downstream mediators in older and younger patients using pre-CP/CPB samples. No significant changes were found in VEGF ($P = .30$), VEGFR2 ($P = .59$), Akt ($P = .87$), phospho-Akt ($P = .74$), and eNOS ($P = .72$) expression between older and younger patients (Figure 2).

Differential Coronary Microvascular Response After CP/CPB

The coronary microvascular response to VEGF was impaired after CP/CPB as compared with before CP/CPB in both older patients ($P = .01$) and younger patients ($P = .02$) (Figure 3, A and B). The coronary microvascular response to SNP was impaired after CP/CPB as compared with before CP/CPB in older patients ($P = .05$) but was slightly increased after CP/CPB in younger patients ($P = .03$) (Figure 3, C and D). The coronary microvascular response to substance P was significantly impaired after CP/CPB as compared with before CP/CPB in older patients ($P < .01$) and tended to decrease after CP/CPB in younger patients, but this trend

TABLE 1. Patient characteristics

Characteristic	Total	Patient age < 70	Patient age $\geq$ 70
No. of patients	42	21	21
Age (y)	68.2 $\pm$ 1.7 (median 69.5)	61.0 $\pm$ 2.6	77.4 $\pm$ 1.3
Gender (M/F)	29/13	14/7	15/6
Hypertension	33	16	17
Hypercholesterolemia	28	16	12
Diabetes	16	11	5
Surgical procedure	CABG 40; CABG + AVR 2	CABG 21	CABG 19; CABG + AVR 2
Aortic crossclamp time (min)	70.8 $\pm$ 4.6	66.9 $\pm$ 6.5	74.8 $\pm$ 6.7
Total CPB time (min)	92.1 $\pm$ 5.3	86.1 $\pm$ 7.5	98.0 $\pm$ 7.5

CABG, Coronary artery bypass grafting; AVR, aortic valve replacement; CPB, cardiopulmonary bypass grafting.

Figure 1. Plot of in vitro response of coronary microvascular response to (A) VEGF ($P = .04$), (B) SNP ($P = .68$), and (C) substance P (*Sub P*) ($P = .59$) using samples obtained before CP/CPB. Solid lines, Younger patients (age <70 years); dotted lines, older patients (age ≥ 70 years). Data are expressed as mean ± SEM. * $P < .05$ versus younger patients. VEGF, vascular endothelial growth factor; SNP, sodium nitroprusside; CP, cardioplegia; CPB, cardiopulmonary bypass.

did not reach statistical significance ($P = .11$) (Figure 3, E and F).

Expression of VEGF-Akt-eNOS Pathways After CP/CPB

In older patients, VEGF protein expression increased after CP/CPB compared with before CP/CPB (1.43-fold, $P < .01$ vs before CP/CPB). Protein expression of VEGFR2 ($P =$

Figure 2. A, Protein expression of VEGF, VEGFR2, Akt, phospho-Akt, and eNOS in human atrial tissue before CP/CPB in patients younger than 70 and older than 70 years analyzed by Western blotting. B, Quantitative analysis of protein expression of VEGF, VEGFR2, Akt, phospho-Akt, and eNOS in human atrial tissue before CP/CPB in patients younger than 70 and older than 70 years. *p-Akt*, Phospho-Akt; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; ENOS, endothelial nitric oxide synthase; CP/CPB, cardioplegia/cardiopulmonary bypass.

.57 vs before CP/CPB), Akt ($P = .78$ vs before CP/CPB), phospho-Akt ($P = .39$ vs before CP/CPB), and eNOS ($P = .79$ vs before CP/CPB) expression was similar in pre- and post-CP/CPB samples (Figure 4).

Circulating CD34⁺ Progenitor Cells

The number of circulating CD34⁺ cells before CP/CPB was similar between younger and older patients (477.5 ± 52.0 vs 465.6 ± 64.6 cells/100 μ L of PB, $P = 0.33$). The number of circulating CD34⁺ cells increased significantly after CP/CPB in younger patients (+190%, $P = .01$). Older patients demonstrated a much smaller increase after CP/CPB, which did not reach statistical significance (124%, $P = .21$). Serum levels of G-CSF increased similarly in younger (12.4 ± 1.7 vs 275.5 ± 69.8 pg/mL, $P < .01$) and older patients after CP/CPB (10.8 ± 1.9 vs 360.0 ± 77.0 pg/mL, $P < .01$) (Figure 5).

Discussion

In this study, we sought to determine the effects of aging on early events important for angiogenesis in humans. The results

Figure 3. Plot of in vitro response of coronary microvascular response to VEGF before and after CP/CPB in patients younger than 70 years ($P = .02$) (A) and older than 70 years ($P = .01$) (B). Plot of in vitro response of coronary microvascular response to SNP before and after CP/CPB in patients younger than 70 years ($P = .03$) (C) and older than 70 years ($P = .05$) (D). Plot of in vitro response of coronary microvascular response to substance P (Sub P) before and after CP/CPB in patients younger than 70 years ($P = .10$) (E) and older than 70 years ($P = .046$) (F). Data are expressed as mean $\pm$ SEM. * $P < .05$ versus before CP/CPB. Solid lines, Before CP/CPB; dotted lines, after CP/CPB. (See Figures 1 and 2 for definitions of abbreviations.)

of this study demonstrate that VEGF-mediated coronary microvascular relaxation is diminished with advanced age. In addition, CP/CPB-induced impairments in coronary microvascular function are accentuated in older patients. Furthermore, mobilization of CD34⁺ stem cell progenitors is reduced after CP/CPB in older patients, despite maintained levels of G-CSF. In summary, advanced age is associated with abnormalities in important early events involved in angiogenesis.

Aging reduces basal and stimulated NO and has been associated with impaired peripheral and coronary endothelial function.^{8,12,13} NO-dependent, flow-mediated, and acetylcholine-induced vasodilatation of the brachial artery is reduced in older individuals as compared with young, healthy volunteers.^{14,16} In this study, we found that coronary microvascular relaxation in response to VEGF was impaired with advanced age. However, the response to substance P, which is mediated

through tachykinin receptor leading to release of NO, was similar between groups. This suggests that older patients may have impaired VEGF signaling in the coronary microcirculation whereas NO availability is unaffected under basal conditions. In response to CP/CPB, coronary microvascular response to VEGF was impaired significantly in all patients. The response to substance P after CP/CPB, however, was reduced significantly and to a greater extent in older patients, suggesting that NO availability was more affected in response to CP/CPB in older patients.

Protein expression in the VEGF-Akt-eNOS signaling pathways was unchanged except for a slight increase in VEGF protein expression after CP/CPB in older patients. This is consistent with our previously published study, which showed that protein expression of VEGF is unchanged in patients with or without coronary artery disease despite differences in cor-

Figure 4. A, Change in protein expression of VEGF, VEGFR2, Akt, phospho-Akt (*p-Akt*), and eNOS in human atrial tissue before and after CP/CPB between patients younger and older than 70 years analyzed by Western blotting. B, Quantitative analysis of the protein expression of VEGF, VEGFR2, Akt, phospho-Akt (*p-Akt*), and eNOS in human atrial tissue before CP/CPB between patients younger and older than 70 years. (See Figures 1 and 2 for definitions of abbreviations.)

onary microvascular response to VEGF.⁸ It is widely established that downstream mediators of VEGF, like Akt and eNOS, are activated and inactivated on the basis of their phosphorylation state.¹⁷ Furthermore, it has been demonstrated that expression of phospho-eNOS is reduced in the aortas of

aged rats.¹⁸ In addition, the activity of eNOS is regulated by subcellular localization and/or protein-protein interactions. For instance, caveolin-1 plays an inhibitory role in eNOS activity.¹⁹ Heat shock protein 90 interacts with eNOS activity by Akt.²⁰ This accumulated evidence demonstrates that the control of VEGF signaling does not occur at the level of protein expression in the VEGF signaling pathway, but may occur at the post-translational level in the human myocardium.

Mobilization of stem cell progenitors is an important factor for tissue repair and angiogenesis that is affected by exposure to the inflammatory stimulus of CP/CPB.^{10,11} Endothelial progenitors are defined as a lineage of cells expressing CD34 and VEGFR2 or AC133.^{10,21} Clinically, CD34 is used as a marker of stem cells, and CD34⁺ cell transplantation has been shown to improve cardiac function in patients with heart failure.²² Recent studies have suggested that the number of circulating endothelial progenitor cells is negatively correlated with age and that mobilization of endothelial progenitors is reduced in elderly patients and those with diabetes mellitus.^{10,21} Furthermore, endothelial progenitor cells appear to have reduced functional activity in elderly patients.¹⁴ In our study, the circulating number of CD34⁺ cells increased significantly in response to CP/CPB by 90% in younger patients after CP/CPB, but only by 24% in older patients. G-CSF, a potent stimulator for stem cell mobilization,²³ increased to a similar extent in both younger and older patients, suggesting that this effect may be independent of G-CSF.

The results in this study provide us some perspective for future angiogenesis therapy in patients with end-stage coronary disease, and particularly in the elderly. We demonstrated that the coronary microvascular response to VEGF is decreased in older patients and that the response is also decreased after CP/CPB in both younger and older patients. Furthermore, previous studies from our laboratory have indicated that the angiogenic response to exogenous VEGF is reduced in the setting of hypercholesterolemic endothelial dysfunction.²⁴ These results suggest that impaired coronary microvascular

Figure 5. Change in circulating CD34⁺ cells after CP/CPB in patients younger than 70 years (A) and older than 70 years (B). CP/CPB, cardioplegia/cardiopulmonary bypass.

response to VEGF, seen in older patients, may be associated with the limited development of angiogenesis in human clinical trials.⁶ We have also focused on strategies to modulate the angiogenic response. For example, we have shown that supplementation with L-arginine (an NO donor) is able to normalize the impaired coronary microvascular response to VEGF as well as to improve the angiogenic response in a chronic ischemia model in hypercholesterolemic swine.²⁵ On the other hand, supplementation with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, atorvastatin, improved endothelial function without improving the angiogenic response to VEGF.²⁶ Therefore, these lines of evidence reinforce the critical role that the endothelium plays in the angiogenic response, but also demonstrate that in addition to endothelial function, there are other factors that also modulate the angiogenic response in vivo. Future strategies at therapeutic angiogenesis should therefore attempt to combat the various antiangiogenic influences that exist in patients with end-stage coronary disease, particularly in the elderly.

Limitations

Our experiments were performed on human atrial tissue samples from patients with coronary artery disease who are potential candidates for angiogenic therapy. Although there are many similarities between atrial and ventricular myocardium, important differences may also exist between the two, and the unavailability of human ventricular tissue constitutes a limitation of this study.^{27,28} In addition, limited sample numbers precluded any subgroup analysis to evaluate the effects of various comorbidities such as diabetes and hypercholesterolemia. Last, although CD34 is an accepted marker for stem progenitors, other lineage makers as well as the functional activity of stem cell progenitors should be further evaluated in future studies.

Conclusions

Advanced age is associated with impaired VEGF signaling as well as greater reductions in NO availability after CP/CPB. Furthermore, progenitor cell mobilization after CP/CPB is diminished in older patients. These age-related differences provide new insights into the limited clinical benefits of angiogenic therapy in certain populations and may serve as future targets that can be modulated to enhance the effects of angiogenic therapy in patients.

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Discussion

Dr Robert M. Mentzer (*Detroit, Mich*). Dr Mieno, I congratulate you and your colleagues on your efforts to help us better understand the impact of aging on VEGF-stimulated angiogenesis. The magnitude of your work should not be underestimated. The use of a preparation that allows one to assess the effect of various stimuli in the presence and absence of VEGF on the microcirculation of human atrial tissue is quite innovative and represents a significant achievement in itself.

The first question I have for you relates to your findings in regard to the intracellular signaling events. As you are aware, there are numerous signaling molecules that have been associated with VEGF. I am curious to learn more about the rationale you used for the selection of the molecules you elected to study, since there are numerous other distant pathway signaling molecules that have been implicated in VEGF-stimulated angiogenesis. These include the mitogen-activated protein kinases, the p38 MAPK, phospholipase C, and protein kinase C.

Dr Mieno. In this study, we looked at specific VEGFR2 signaling pathways, because the VEGFR2 signaling pathway is more important for the development of angiogenesis than the VEGFR1 signaling pathways and other signaling pathways such as protein kinase C and those you have suggested. We have not looked at other signaling pathways.

Dr Mentzer. In regard to the experimental design, it would be helpful if you would comment on the challenges and limitations of studying both aging and VEGF in the context of using CP/CPB. Since this setting introduces a number of complex variables, is it likely that your findings reflect observations that are limited to this setting? Would you also comment on some of the contributing factors that might be considered unique to CP/CPB?

Dr Mieno. I think CP/CPB is important to induce ischemic or oxidative stress. Recent publications demonstrated that VEGF signaling is altered by ischemia and other kinds of stress. I think CP/CPB is one of the activators of VEGF signaling pathways.

It is easy to get human atrial tissue before and after CPB. And CP/CPB also induces ischemia or reperfusion injury in tissue. That is why we are looking at difference in VEGF signaling pathway before and after CPB.

Dr Mentzer. Finally, I would like to hear your thoughts on why the results with VEGF in the surgical setting have been

disappointing. Five years ago there was considerable enthusiasm for VEGF therapy. At this very organization there were reports of both preclinical studies and phase I clinical trials that seemed to indicate a very promising role for this growth factor. The results suggested improvement in ventricular function and, at the very least, relief from disabling angina. As you pointed out in your first slide, this no longer appears to be the case. On the basis of the preclinical work presented today, what does the future hold for us as it relates to VEGF gene therapy? What are your thoughts as to where we should go from here?

Dr Mieno. As shown in this presentation, mobilization response and coronary microvascular relaxation are important factors for development of angiogenesis. We demonstrated that events such as coronary microvascular relaxation and the progenitor mobilization response were impaired by aging as well as after CPB. This impaired response may be associated with limited development of angiogenesis in human clinical trials using the VEGF protein. In the clinical setting, if we try to perform cell and protein therapy, some additional treatment to improve the coronary microvascular relaxation may be required in advance. Previously, we have demonstrated that L-arginine supplementation, an NO donor, is able to normalize the impaired coronary microvascular response in ischemic left ventricular tissues of swine models of chronic ischemia.

Dr Sellke. Maybe I can add that there are some intrinsic problems with the myocardium in patients requiring angiogenic therapy. zHypercholesterolemia, diabetes, oxidative stress, and other factors affect the effectiveness of the angiogenic process. Protein growth factors do not work, nor does gene therapy work well in these patients. We have to overcome these inhibitory factors before gene therapy or other types of angiogenic therapy will become effective.

Dr Guo-Wei He (*Hong Kong, China*). There have been studies showing that there are two subtypes of VEGF receptors, predominantly on the vascular endothelial cell, KDR receptors (VEGFR2) and Flt receptors (VEGFR1). You have not used antagonists for either KDR or Flt receptors. Could you please comment on why you did not do it or whether you plan to do that.

Second, the diminished response of the microvascular circulation to VEGF could be due to two reasons: (1) endothelial response reduced or (2) smooth muscle cell response reduced. Which one do you think is the factor in the patients with impaired VEGF response?

Dr Mieno. The first question is, as you suggested, that there are two major signaling pathways, VEGFR1 and VEGFR2, but we have not differentiated the specific signaling pathways in coronary microvascular relaxation.

Dr He. Is VEGFR2 related to KDR or related to Flt?

Dr Mieno. We have not used specific antibodies like VEGFR1 or VEGFR2, so I am not sure which pathway is predominantly involved in the coronary microvascular relaxation to VEGF.

Dr He. My second question is, is it due to the diminished response of the endothelial cell or smooth muscle cell?

Dr Mieno. The reduced coronary microvascular relaxation is due to endothelial dysfunction as well as smooth muscle dysfunction. I did not show the data for coronary microvascular relaxation to SNP because of the time limitation, but we have those data. The coronary microvascular relaxation to SNP was impaired in older patients after CPB. This suggests smooth muscle cell dysfunction.