Endothelium-derived microparticles inhibit angiogenesis in the heart and enhance the inhibitory effects of hypercholesterolemia on angiogenesis

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Ou ZJ, Chang FJ, Luo D, Liao XL, Wang ZP, Zhang X, Xu YQ, Ou JS. Endothelium-derived microparticles inhibit angiogenesis in the heart and enhance the inhibitory effects of hypercholesterolemia on angiogenesis. Am J Physiol Endocrinol Metab 300: E661–E668, 2011. First published January 18, 2011; doi:10.1152/ajpendo.00611.2010.—Therapeutic angiogenesis remains unsuccessful in coronary artery disease. It is known that plasma endothelium-derived microparticles (EMPs) are increased in coronary artery disease and that hypercholesterolemia can inhibit angiogenesis. We evaluated the relationship between EMPs and hypercholesterolemia in the impairment of angiogenesis. EMPs isolated from human umbilical vein endothelial cells were injected into low-density lipoprotein receptor-null (LDLr−/−) mice fed a Western diet for 2 wk and C57BL6 mice for 6 h or were directly added to the tissue culture media. Hearts isolated from mice were sectioned and cultured, and endothelial tube formation was measured. The expression and phosphorylation of endothelial NO synthase (eNOS) and the generation of NO in the hearts were determined. Angiogenesis was inhibited by pathophysiological concentrations of EMPs but not physiological concentrations of EMPs in hearts from C57BL6 mice. However, angiogenesis was inhibited by EMPs at both physiological and pathophysiological concentrations of EMPs in hearts from hypercholesterolemic LDLr−/− mice. Pathophysiological concentrations of EMPs decreased eNOS phosphorylation at Ser1177 and NO generation without altering eNOS expression in hearts from C57BL6 mice. Both physiological and pathophysiological concentrations of EMPs decreased not only eNOS phosphorylation at Ser1177 and NO generation, but eNOS expression in hypercholesterolemic hearts from LDLr−/− mice. These data demonstrated that pathophysiological concentrations of EMPs could inhibit angiogenesis in hearts by decreasing eNOS activity. EMPs and hypercholesterolemia mutually enhanced their inhibitory effect of angiogenesis by inducing eNOS dysfunction. Our findings suggest a novel mechanism by which hypercholesterolemia impairs angiogenesis.

Angiogenesis has been identified as a potential therapy for myocardial ischemia due to coronary artery disease. Many studies in animal models have successfully increased angiogenesis in the heart (13, 38, 43). However, clinical trials have failed to demonstrate that therapeutic angiogenesis is as effective in patients (14, 40, 41). Studies suggested that endothelial dysfunction and decreased generation of nitric oxide (NO) may be one of the major reasons for the poor effectiveness of angiogenesis in patients. Therefore, the National Institutes of Health called for more basic research to determine the actual mechanisms of inhibition of angiogenesis (1).

Although coronary artery disease is a complex disease with multiple etiologies and aggravating events, the link between hypercholesterolemia (elevated plasma cholesterol levels) and coronary artery disease has been well established (12, 18, 24). It is generally recognized that angiogenesis is impaired in hypercholesterolemia (40, 42, 46). However, the actual mechanism of inhibition of angiogenesis by hypercholesterolemia remains unclear.

EMP have been shown to inhibit angiogenesis in cell culture models (19, 27); however, there is no evidence available to show whether EMPs inhibit angiogenesis in the heart. We and other investigators (5, 9, 47) have demonstrated that EMPs could reduce NO generation and induce endothelial dysfunction. Interestingly, Ferreira et al. (11) showed that even a single high-fat meal could significantly induce an increase in the level of plasma EMPs in humans. Because the plasma levels of EMPs are increased in coronary artery disease and acute coronary syndrome, it is possible that an increased level of EMPs may be one of the major factors leading to the poorer therapeutic effectiveness of angiogenesis in human patients compared with that in normal healthy animal models (2, 3, 20, 25, 30, 51). Thus, in this study we separated our study into two parts; the first part used C57BL6 mice to test whether EMPs inhibit heart angiogenesis, and the second part used LDLr−/− mice fed a Western diet to evaluate the relationship between EMPs and hypercholesterolemia in the impairment of angiogenesis.

MATERIALS AND METHODS

Generation of EMPs. EMPs were generated by incubating human umbilical vein endothelial cells (HUVECs) with plasminogen-activated inhibitor-1 (PAI-1; American Diagnostica, Stamford, CT) as described earlier (5, 9, 35, 37). Briefly, passage 4 HUVECs were grown to confluence in T75 flasks coated with 1% gelatin in EGM-2 medium (Clonetics, East Rutherford, NJ) containing 20% fetal bovine serum. Cultured cells were maintained at 37°C in 5% humidified CO2. At the time of EMPs generation, the cells were washed three times with HBSS and serum starved at 37°C in nonsupplemented EBM-2 (Clonetics) for 2 h. After serum starvation, cells were washed again
EMPs inhibit heart angiogenesis

Animal experiments. All animal experiments were approved by The First Affiliated Hospital, Sun Yat-sen University Review Board and Animal Research Committee. The investigation conformed to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Eight-week-old male C57BL6 mice were obtained from the animal center of Sun Yat-sen University, northern campus. Eight-week-old male low-density lipoprotein receptor-null mice (LDLr<sup>−/−</sup>) were from Jackson Laboratory (Bar Harbor, ME). LDLr<sup>−/−</sup> mice were fed a Western diet for 2 wk (32). LDLr<sup>−/−</sup> mice were fed a chow diet as the control of hypercholesterolemic LDLr<sup>−/−</sup> mice.

Endothelial cell tube formation. Murine hearts were isolated from the C57BL6 and LDLr<sup>−/−</sup> mice and stored in sterile MOPS buffer at 4°C. Immediately, the hearts were cut into thin slices (100–160 μm) as described (50). The heart sections were placed in fibrin-coated 24-well cluster plates, allowed to adhere, and incubated in 10% FBS-DMEM medium containing 2% antibiotics-antimycotics. Culture media were supplemented with vascular endothelial growth factor (VEGF, 50 ng/ml final concentration) and PBS or different concentrations of EMPs and then incubated for 5–7 days. Endothelial cell (EC) tube formation was quantified after day 5, as described (49).

Another set of C57BL6 and LDLr<sup>−/−</sup> mice were first injected with PBS or different concentrations of EMPs via the dorsal penile vein. After 6 h, the hearts were isolated and sectioned and cultured as described above except without incubation in the presence of EMPs. EC tube formation was quantified after day 5, as described (49).

Measurement of NO generation in hearts. C57BL6 and LDLr<sup>−/−</sup> mice were first injected with different concentrations of EMPs via the dorsal penile vein. After 6 h, the hearts were isolated and sectioned and cultured as described above except without incubation in the presence of EMPs. NO generation in hearts was quantified after 24 h with 30-s intervals between homogenizations. The homogenates were pieces with an iris scissors and homogenized five times on ice (10 s each). The homogenates were then centrifuged at 2,000 rpm for 8 min at 4°C. The supernatant was transferred to a cold microcentrifuge tube, and protein concentrations were determined by a bicinchoninic acid protein assay. The protein was used for Western blot analysis. The expression of eNOS in the heart was tested whether injection of EMPs into the circulation of the C57BL6 mice significantly inhibited the formation of EC tubes in cultured sections of hearts isolated from C57BL6 mice in medium containing VEGF and EMPs at 10<sup>5</sup>/ml was not impaired or inhibited. In contrast, generation of EC tubes from the thin sections of hearts isolated from C57BL6 mice were short with little body in medium containing VEGF and EMPs at 10<sup>4</sup>/ml was not impaired or inhibited. Inhibition of angiogenesis from the cultured sections of hearts stimulated with VEGF and EMPs at 10<sup>4</sup>/ml significantly inhibited the formation of EC tubes even more dramatically (Fig. 1C). Quantitative studies demonstrated that concentrations of 10<sup>5</sup> and 10<sup>6</sup> EMPs/ml significantly inhibited the formation of EC tubes cultured sections of hearts from C57BL6 mice stimulated with VEGF, and EMPs at 10<sup>4</sup>/ml inhibited EC tube formation even more dramatically (Fig. 1E).

EMPs inhibit angiogenesis of hearts ex vivo. Next, we tested whether injection of EMPs into the circulation of the mice also impaired the angiogenic responses from the hearts ex vivo. Previously, we (9) showed that EMPs induced acute lung injury 6 h after injection into the circulation. Therefore, we chose to isolate the hearts from the C57BL6 mice for sectioning and cultured with VEGF at 6 h after injection with different concentrations of EMPs. As shown in Fig. 2, injection of concentrations of 10<sup>5</sup> and 10<sup>6</sup> EMPs/ml significantly inhibited the formation of EC tubes cultured sections of hearts stimulated with VEGF, revealing that EMP3 impaired angiogenesis from the hearts ex vivo.
the NO concentration in isolated hearts at 6 h after the mice had been injected with different concentrations of EMPs. Injection of a concentration of 10^5 but not 10^4 EMP/ml into the circulation of C57BL6 mice significantly decreased NO generation (Fig. 3).

**EMPs decreased eNOS phosphorylation without altering expression of eNOS in hearts.** To determine the mechanism by which EMPs inhibited NO generation, expression and phosphorylation of eNOS in the hearts were evaluated. As shown in Fig. 4, injection of a concentration of 10^5 but not 10^4 EMP/ml into the circulation of C57BL6 mice significantly decreased eNOS phosphorylation at the Ser^1177 site. However, concentrations of neither 10^5 nor 10^4 EMP/ml significantly altered eNOS expression in the hearts.

**EMPs within physiological concentrations inhibited angiogenesis in cultured sections of hypercholesterolemic hearts.** The study described above showed that pathophysiological but not physiological concentrations of EMPs impaired angiogenesis in the hearts. It is well known that hypercholesterolemia impairs angiogenesis (40, 42, 46). Thus, we further explored whether hypercholesterolemia and EMPs had a synergistic effect in impairing angiogenesis in the heart. LDLr^{-/-} mice were fed a Western diet or a chow diet for 2 wk. Hearts from the LDLr^{-/-} mice were isolated, sliced, and cultured with VEGF and different concentrations of EMPs as described above. In our preliminary experiments, we found that EC tube formation from the hearts of LDLr^{-/-} mice fed a Western diet for 2 wk still occurred normally. However, if LDLr^{-/-} mice...
were fed a Western diet for more than 2 wk, the angiogenic response from the hearts was impaired (data not shown). Thus, we determined that a feeding duration of 2 wk would allow us to investigate whether physiological concentrations of EMPs could also inhibit angiogenesis during hypercholesterolemia.

Total cholesterol levels in the plasma from LDLr−/− mice fed a Western diet for 2 wk (656.8 ± 116.8 mg/dl) was significantly increased compared with that of C57BL/6 mice (56.9 ± 7.5 mg/dl, P < 0.05) and that of LDLr−/− mice fed a chow diet (137.1 ± 22.5 mg/dl, P < 0.05). EC tube formation from cultured sections of the hearts from LDLr−/− mice fed with a Western diet for 2 wk and stimulated with VEGF was not significantly different compared with that of the hearts from LDLr−/− mice fed a chow diet and stimulated with VEGF (Fig. 5). However, EC tube formation from cultured sections of the hearts from hypercholesterolemic LDLr−/− mice stimulated with VEGF and treated with EMPs at 10⁴/ml was significantly inhibited compared with that of the hearts from hypercholesterolemic LDLr−/− mice stimulated only with VEGF and without EMPs (Fig. 5). EMPs at 10⁴/ml inhibited EC tube formation even more dramatically (Fig. 5), indicating that hypercholesterolemia enhanced the antiangiogenesis effect of EMPs in the heart.

**EMPs within physiological concentrations inhibited angiogenesis of hypercholesterolemic hearts ex vivo.** As physiological concentrations of EMPs could impair the angiogenic responses from hypercholesterolemic mouse hearts in vitro, we further tested whether injection of physiological concentrations of EMPs into the circulation of hypercholesterolemic LDLr−/− mice also impaired the angiogenic responses from the hearts ex vivo as was done with the C57BL/6 mice above. EC tube formation was significantly impaired in cultured sections of hearts from hypercholesterolemic LDLr−/− mice injected with 10⁴ or 10⁵ EMP/ml and stimulated with VEGF compared with that of the hearts from LDLr−/− mice fed either a Western diet or a chow diet and stimulated only with VEGF (Fig. 6). Taken together, these data suggest that hypercholesterolemia and EMPs have a synergetic effect in impairing angiogenesis.

EMPs decreased NO generation in hypercholesterolemic hearts. We next measured the concentration of NO in hearts isolated from LDLr−/− mice at 6 h after injection with PBS or 10³ or 10⁴ EMP/ml. As shown in Fig. 7, NO generation in the hearts from LDLr−/− mice fed a Western diet for 2 wk was not significantly different from that of LDLr−/− mice fed a chow diet. However, injection of EMPs into hypercholesterolemic LDLr−/− mice had a dose-dependent effect in decreasing NO generation in the hearts.

EMPs within physiological concentrations decreased eNOS phosphorylation and expression in hypercholesterolemic hearts. Finally, we examined the expression and phosphorylation of eNOS in hearts from LDLr−/− mice. Figure 8 shows that LDLr−/− mice fed a Western diet for 2 wk slightly but not significantly increased eNOS expression and slightly decreased eNOS phosphorylation at the Ser1177 site in the hearts compared with LDLr−/− mice fed a chow diet. Injection of both 10³ and 10⁴ EMP/ml into the circulation of hypercholesterolemic LDLr−/− mice not only significantly decreased eNOS phosphorylation at Ser1177 but also decreased eNOS expression (Fig. 8).

**DISCUSSION**

The results of the fibrin gel assay for angiogenesis in cultured sections of hearts in vitro and ex vivo clearly indicated that angiogenic responses to VEGF stimulation were impaired in both normal and hypercholesterolemic mouse hearts that were either incubated with or exposed to EMPs in the circulation. Our findings are consistent with the reports by Mezentsev et al. and Klinkner et al. that EMPs impaired endothelial tube formation in a cell culture model in vitro (19, 27). Although we chose cultured sections of hearts for the in vitro and ex vivo models, our findings suggest that EMPs may impair angiogenesis from the heart in vivo, which needs to be further confirmed in future studies. This study provides the first
direct evidence that EMPs impair tissue angiogenesis, which is similar to angiogenesis in vivo.

Our data show that only pathophysiological concentrations of EMPs can impair angiogenesis in hearts from C57BL6 mice. This indicates that the angiogenic responses in the heart are normal if the circulating levels of EMPs are not elevated but that angiogenesis can be impaired once the circulating levels of EMPs are increased upon activation. These findings also suggest angiogenesis may be inhibited by EMPs at physiological concentrations in cultured sections of hypercholesterolemic hearts. EC tube formation from cultured sections of hearts from low-density lipoprotein receptor-null (LDLr⁻/⁻) mice fed a Western diet (WD) for 2 wk was significantly different from that of LDLr⁻/⁻ mice fed a chow diet when stimulated with VEGF. However, EC tube formation from cultured sections of hearts from LDLr⁻/⁻ mice fed WD for 2 wk was significantly inhibited when stimulated with VEGF and treated with concentrations of 10⁴ and 10⁵ EMP/ml in culture medium. Bar chart shows quantitative data for EC tube formation (*10⁴ EMP/ml, 10⁵ EMP/ml vs. PBS, *P < 0.05). Data represent means ± SE; n = 8.

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Fig. 4. EMPs decrease endothelial NO synthase (eNOS) phosphorylation (p) without altering eNOS expression in hearts. Phosphorylation of eNOS at Ser1177 was significantly decreased in hearts from C57BL6 mice injected with a concentration of 10⁵ but not 10⁴ EMP/ml. However, no significant difference was found in expression of eNOS among all groups. Bar chart shows relative band densities of the blots (*10⁵ EMP/ml vs. PBS, *P < 0.05). Data represent means ± SE; n = 6.

Fig. 5. EMPs at physiological concentrations inhibit angiogenesis in cultured sections of hypercholesterolemic hearts. EC tube formation from cultured sections of hearts from LDLr⁻/⁻ mice fed a Western diet (WD) for 2 wk was significantly different from that of LDLr⁻/⁻ mice fed a chow diet when stimulated with VEGF. However, EC tube formation from cultured sections of hearts from LDLr⁻/⁻ mice fed WD for 2 wk was significantly inhibited when stimulated with VEGF and treated with concentrations of 10⁴ and 10⁵ EMP/ml in culture medium. Bar chart shows quantitative data for EC tube formation (*10⁴ EMP/ml, 10⁵ EMP/ml vs. PBS, *P < 0.05). Data represent means ± SE; n = 8.

Fig. 6. EMPs at physiological concentrations inhibited angiogenesis of hypercholesterolemic hearts ex vivo. EC tube formation from cultured sections of hearts from LDLr⁻/⁻ mice fed WD for 2 wk and injected with PBS was no significantly different from that of LDLr⁻/⁻ mice fed Chow and injected with PBS when stimulated with VEGF. EC tube formation from cultured sections of hearts from LDLr⁻/⁻ mice fed WD for 2 wk and injected with concentrations of 10⁴ or 10⁵ EMP/ml was significantly inhibited when stimulated with VEGF in culture medium. Hearts were isolated and sectioned 6 h after injection of the mice with EMPs (*10⁴ EMP/ml, 10⁵ EMP/ml vs. PBS, *P < 0.05). Bar chart shows quantitative data for EC tube formation. Data represent means ± SE; n = 8.
that the plasma EMP level was already increased under hypercholesterolemia. Another possible explanation is that plasma EMPs, enhanced endothelial dysfunction, which resulted in the small injury, either exposure to injected EMPs or culture with already insulted by the hypercholesterolemia and that even a single high-fat meal could significantly increase the plasma levels of EMPs in humans. Thus, any small increase in the levels of EMPs in the circulation by injection or activation will affect angiogenesis in the heart. However, the observation that angiogenesis was also impaired in cultured sections of hypercholesterolemic hearts with physiological concentrations of EMPs supports the first possibility that EMPs enhanced the endothelial dysfunction in hypercholesterolemia, which led to the inhibition of angiogenesis in the hearts. Future study should examine whether physiological concentrations of EMPs will also impair angiogenesis in other diseases in which the EMPs level is increased and the endothelium is dysfunctional.

There is substantial evidence that effective angiogenesis requires NO (10, 28, 46, 54). We and other researchers (5, 9) have shown that EMPs decrease NO production in endothelial cell culture. Here, we found decreased generation of NO in hearts from C57BL6 mice injected with pathophysiological concentrations of EMPs, indicating that EMPs may impair angiogenesis by decreasing NO production. In addition, we also found decreased generation of NO in hearts from hypercholesterolemic LDLr<sup>−/−</sup> mice injected with even physiological concentrations of EMPs. These findings also confirm that decreased generation of NO may be an important factor that in many diseases in which plasma levels of EMPs are elevated.

Angiogenesis is a crucial therapy for treating myocardial ischemia due to coronary artery disease. Hypercholesterolemia is a major factor causing coronary artery disease. It has been reported that angiogenesis is impaired by hypercholesterolemia and that plasma levels of EMPs are increased in coronary artery disease and acute coronary syndrome (2, 3, 20, 25, 30, 51). Thus, we further investigated the relationship between hypercholesterolemia and EMPs as it pertains to the inhibition of angiogenesis. To avoid inhibition of angiogenesis by hypercholesterolemia itself, LDLr<sup>−/−</sup> mice were fed a Western diet for only 2 wk. Such a feeding interval raised the total cholesterol in the plasma to hypercholesterolemic levels (52), but the angiogenic responses to VEGF stimulation remained normal in our preliminary experiments. It is possible that hypercholesterolemia did not obviously induce endothelial dysfunction in a such short time, which allowed us to investigate whether physiological concentrations of EMPs could inhibit angiogenesis during hypercholesterolemia. We found that, even at physiological concentrations of EMPs, angiogenesis of the heart was impaired in hypercholesterolemia.

We speculate that the antiangiogenesis effect may have occurred at physiological concentrations of EMPs under hypercholesterolemic conditions because the endothelium was already insulted by the hypercholesterolemia and that even a small injury, either exposure to injected EMPs or culture with EMPs, enhanced endothelial dysfunction, which resulted in the impairment of angiogenesis. Another possible explanation is that the plasma EMP level was already increased under hypercholesterolemic conditions and injection of even a physiological concentration of EMPs increased the plasma concentration to pathophysiological levels. This hypothesis is supported by the results of the study by Ferreira et al. (11), which showed even a single high-fat meal could significantly increase the plasma levels of EMPs in humans. Thus, any small increase in the levels of EMPs in the circulation by injection or activation will affect angiogenesis in the heart.
impairs angiogenesis in hypercholesterolemia. Such findings support the idea that endothelial dysfunction and decreased NO generation in coronary artery disease may play a crucial inhibitory role in the response to exogenous angiogenic agents, as EMPs have been shown to induce endothelial dysfunction (1, 46). These data indicate that hypercholesterolemia may increase EMPs, thus inducing endothelial dysfunction and decreasing NO generation to inhibit angiogenesis in the heart.

One of the enzymes involved in the synthesis of NO is eNOS, which is critical for angiogenesis (53). Therefore, we further tested the expression and phosphorylation of eNOS in hearts from mice injected with EMPs. We found that eNOS phosphorylation at Ser1177 was significantly decreased by pathophysiological concentrations of EMPs in nonhypercholesterolemic hearts and by physiological or pathophysiological concentrations of EMPs in hypercholesterolemic hearts. Unexpectedly, eNOS expression was decreased only in hypercholesterolemic mice and not in nonhypercholesterolemic mice injected with either physiological or pathophysiological concentrations of EMPs. These findings demonstrated that EMPs inhibited angiogenesis by inhibiting the eNOS pathway. A previous study demonstrated that eNOS expression was increased in hypercholesterolemia, which is consistent with our current findings that eNOS expression was slightly increased in LDLR−/− mice fed a Western diet for 2 wk (8, 33, 34). Our data showing that eNOS expression was decreased was injected in hypercholesterolemic hearts when EMPs were present indicated that endothelial cells were quite harmful under such conditions and did not respond to exogenous angiogenic agents. These findings may explain why therapeutic angiogenesis in patients with coronary artery diseases is less effective than in normal, healthy animal models (14, 40, 41). In addition, our data showed that physiological concentrations of EMPs did not inhibit heart angiogenesis in nonhypercholesterolemic mice but did inhibit heart angiogenesis in hypercholesterolemic LDLR−/− mice with decreasing eNOS expression, suggesting that hypercholesterolemia also enhanced EMPs inhibitory effects on angiogenesis by inducing eNOS dysfunction.

In summary, our findings indicate that EMPs inhibited angiogenesis in the heart by inhibiting the eNOS pathway to decrease generation of NO. EMPs and hypercholesterolemia mutually enhanced their inhibitory effect of angiogenesis. Reducing the number of EMPs in the circulation or blocking their effects may be an effective therapeutic approach for treating myocardial ischemia due to coronary artery disease.

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DISCLOSURES

No conflicts of interest are reported by the authors.

REFERENCES


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