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

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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.

EMPs 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 activator-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

and stimulated with 5 ml of EBM-2 supplemented with 10 ng/ml human PAI-1. Three hours later, the EMP-rich supernatant was collected and centrifuged (300 g, 10 min) to remove cell debris. The supernatant was removed after ultracentrifugation (10^5 g, 60 min), and EMPs were resuspended in PBS at room temperature for experimentation.

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-/-) were from Jackson Laboratory (Bar Harbor, ME). LDLR-/- mice were fed a Western diet for 2 wk (32). LDLR-/- mice were fed a chow diet as the control of hypercholesterolemic LDLR-/- mice.

Endothelial cell tube formation. Murine hearts were isolated from the C57BL6 and LDLR-/- mice and stored in sterile MOPS buffer at 4°C. Immediately, the hearts were cut into thin slices (100–160 mm) 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 2X 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-/- 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-/- 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. EC tube formation was quantified after day 5, as described (49).

Western blot analysis. C57BL6 and LDLR-/- mice were first injected with different concentrations of EMPs via the dorsal penile vein. After 6 h, the hearts were isolated and frozen in liquid nitrogen for Western blot analysis. The expression of eNOS in the hearts was determined as described previously (21, 22, 31). Briefly, frozen heart samples were pulverized and placed in a modified RIPA buffer (21). The mixture was then homogenized and sonicated to break the cells, and the cell debris was removed by centrifugation at 14,000 g for 10 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 measured as micromoles per gram of protein.

Statistical analysis. Differences among test groups were analyzed by ANOVA, using Newman-Keuls multiple comparison test (Prism 4.0; GraphPad Software, San Diego, CA). A P value of <0.05 was considered statistically significant.

RESULTS

EMPs inhibit angiogenesis in cultured sections of heart. For the fibrin gel assay of angiogenesis, hearts were cut into thin slices and cultured to observe the formation of EC tubes. Normally, EC tubes emanate from the cultured sections of hearts on day 2–3 and form some long EC tubes on day 5–7 when the culture medium contains VEGF. Figure 1A shows a long EC tube from a cultured section of the hearts isolated from C57BL6 mice in medium containing VEGF and EMPs at 10^5/ml and 10^6/ml, indicating a good angiogenic response to VEGF stimulation in the normal heart. Similar formation of EC tubes is shown in Fig. 1B, suggesting that angiogenesis from the cultured sections of hearts isolated from C57BL6 mice in medium containing VEGF and EMPs at 10^5/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 10^5/ml and 10^6/ml, hinting that the angiogenic response was inhibited (Fig. 1, C and D). Quantitative studies demonstrated that concentrations of 10^5 and 10^6 EMP/ml significantly inhibited the formation of EC tubes in cultured sections of hearts from C57BL6 mice stimulated with VEGF, and EMPs at 10^5/ml inhibited EC tube formation even more dramatically (Fig. 1E).

EMPs inhibited 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 culture with VEGF at 6 h after injection with different concentrations of EMPs. As shown in Fig. 2, injection of concentrations of 10^5 and 10^6 EMP/ml inhibited the formation of long EC tube from a cultured section of the hearts isolated from C57BL6 mice in medium containing VEGF and EMPs at 10^5/ml and 10^6/ml, hinting that the angiogenic response was inhibited (Fig. 2, A and B). Quantitative studies demonstrated that concentrations of 10^5 and 10^6 EMP/ml significantly inhibited the formation of EC tubes in cultured sections of hearts from C57BL6 mice stimulated with VEGF, and EMPs at 10^5/ml and 10^6/ml inhibited EC tube formation even more dramatically (Fig. 2E).

A previous study showed that concentrations of EMPs only at 10^5/ml or more, but not 10^4/ml, could inhibit tube formation in a cell culture model (27) and induce endothelial dysfunction (5). Our present data are consistent with these previous findings. In addition, it has been reported that the physiological concentrations of EMPs was ~10^6/ml (4, 17), and in cardiovascular diseases, EMPs increased to 10^5/ml, which represents pathophysiological concentrations (36, 44, 45). Therefore, we chose 10^5/ml or 10^6/ml of EMPs in our next experiments.

EMPs decreased NO generation in hearts. To determine whether EMPs affected the NO level in hearts, we measured obtained with an EPSON scanner and Adobe Photoshop CS. Densities of the bands were quantified from the scanned images with NIH Image 1.63 software. Relative changes of eNOS in the lysates were determined as follows. The band densities for eNOS were divided by the band densities for the corresponding β-actin. The band densities for P-eNOS S1177 were divided by the band densities for the corresponding eNOS. The resulting ratios of P-eNOS S1177 to eNOS and eNOS to β-actin for the controls were used to calculate relative ratios for all experimental groups after setting the control ratio to 1. Data are represented means ± SE.
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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice fed a Western diet for 2 wk was not significantly different compared with that of the hearts from LDLr<sup>−/−</sup> mice fed a chow diet (P 0.05). Data represent means ± SE; n = 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.

Together, these data suggest that hypercholesterolemia and EMPs have a synergistic effect in impairing angiogenesis.

**EMPs decreased NO generation in hypercholesterolemic hearts.** We next measured the concentration of NO in hearts isolated from LDLr<sup>−/−</sup> mice at 6 h after injection with PBS or 10<sup>4</sup> or 10<sup>5</sup> EMP/ml. As shown in Fig. 7, NO generation in the hearts from LDLr<sup>−/−</sup> mice fed a Western diet for 2 wk was not significantly different from that of LDLr<sup>−/−</sup> mice fed a chow diet. However, injection of EMPs into hypercholesterolemic LDLr<sup>−/−</sup> 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<sup>−/−</sup> mice. Figure 8 shows that LDLr<sup>−/−</sup> mice fed a Western diet for 2 wk slightly but not significantly increased eNOS expression and slightly decreased eNOS phosphorylation at the Ser<sup>1177</sup> site in the hearts compared with LDLr<sup>−/−</sup> mice fed a chow diet. Injection of both 10<sup>5</sup> and 10<sup>6</sup> EMP/ml into the circulation of hypercholesterolemic LDLr<sup>−/−</sup> mice not only significantly decreased eNOS phosphorylation at Ser<sup>1177</sup> but also decreased eNOS expression (Fig. 8).

**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<sup>−/−</sup> 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<sup>−/−</sup> mice injected with 10<sup>5</sup> or 10<sup>6</sup> EMP/ml and stimulated with VEGF compared with that of the hearts from LDLr<sup>−/−</sup> 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 synergistic effect in impairing angiogenesis.

**Fig. 3.** EMPs decrease NO generation in hearts. A significant decrease in NO concentration was evident in hearts isolated 6 h after injection with PBS or 10<sup>4</sup> or 10<sup>5</sup> EMP/ml (*10<sup>5</sup> EMP/ml vs. PBS, *P < 0.05). Data represent means ± SE; n = 12.
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.
that the plasma EMP level was already increased under hypercholesterolemia. Another possible explanation is that 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−/− 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−/− 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.

In our preliminary experiments, it was observed that angiogenesis of the heart was impaired in hypercholesterolemia. We found that, even at physiological concentrations of EMPs, angiogenesis of the heart was impaired in hypercholesterolemia. 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.

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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 in hypercholesterolemic hearts when EMPs were present indicated that endothelial cells were quite harmful under such conditions and supported the idea that endothelial dysfunction and decreased NO are related to the development of atherosclerosis.

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.

EMPHS INHIBIT HEART ANGIOGENESIS

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