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VASCULAR ENDOTHELIAL GROWTH FACTOR CAUSES ENDOTHELIAL PROLIFERATION AFTER VASCULAR INJURY

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SUMMARY: Vascular endothelial growth factor was infused into rat carotid arteries for 3 minutes immediately after endothelial denudation by balloon injury. Endothelial proliferation was determined by immunohistochemical labelling of proliferating cell nuclear antigen using Häutchen preparations. The proliferation index, or number of proliferating cells/total cells, measured at 25.5 or 30 hours was markedly increased after infusion of vascular endothelial growth factor. In addition, the total number of proliferating cells increased with increasing doses up to 100μg total dose per infusion. These data indicate that infusion of vascular endothelial growth factor increases endothelial cell proliferation after mechanical denudation injury of the vascular wall.

Loss of the endothelium, such as occurs after balloon angioplasty, is associated with increased smooth muscle cell proliferation and neointimal formation, processes that often lead to restenosis after coronary angioplasty (1). Regeneration of the endothelial layer may be important in limiting neointimal formation and smooth muscle cell (SMC) growth after vascular injury (1,2,3). However, the factors responsible for endothelial proliferation and regeneration after denudation injury remain unclear.

Vascular endothelial growth factor (VEGF) induces endothelial proliferation *in vitro*. Increased synthesis of VEGF, as seen with tumors, and with cardiac and retinal ischemia, is associated with angiogenesis(4,5,6,7). In other models of tissue injury there is evidence to suggest VEGF plays a role in wound repair. For example, dermal wounding resulted in increased VEGF synthesis in rats and guinea pigs (8).

Infusion of VEGF has been shown to increase angiogenesis in the rabbit hindlimb after severe ischemia, resulting in increased blood vessel development and flow (9). However, it is not known if VEGF stimulates endothelial proliferation after physiological

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Abbreviations: SMC, smooth muscle cells; VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; TBS, tris-buffered saline; bFGF, basic fibroblast growth factor; TBF-ß1, transforming growth factor beta-1.

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injury of an existing monolayer within the vascular wall. In order to address this issue, we infused recombinant human $VEGF_{165}$ into rat carotid arteries following balloon denudation. We chose this variant of VEGF as it is a form normally secreted from VEGF-producing cells and is found in high abundance (10,11). Endothelial proliferation was determined using immunohistochemical labelling of proliferating cell nuclear antigen (PCNA) on Häutchen preparations of the arteries (12,13). Our data show that VEGF infusion increased the total number of proliferating endothelial cells and extended the area of vessel wall involved in proliferation.

METHODS

Mechanical Denudation: Male Sprague-Dawley rats (400-500g) were anesthetized with halothane inhalation. The left common carotid artery was surgically exposed and 1.5 cm of that artery was denuded of endothelium using a 2F Fogarty balloon embolectomy catheter. The balloon catheter was inserted through the external carotid artery, inflated 3 times, rotated several times at each inflation, and then removed. The proximal common carotid artery was clamped to block blood flow and recombinant human $VEGF_{165}$, a generous gift of N. Ferrara (Genentech, S. San Francisco), was infused via a catheter (PE 10 tubing) into the carotid artery for 3 minutes in various concentrations in a total volume of 1 ml vehicle solution (phosphate-buffered saline (PBS, 0.1M) with 0.1% bovine serum albumin at pH 7.25) (9). Control animals were infused with vehicle alone. Blood flow was restored, the wound was closed and the animals were allowed to recover.

Carotid Removal for Analysis: To verify the extent and location of denudation, one hour prior to sacrifice the animals were injected with 0.5 ml of 5% Evans blue dye via tail vein injection (14). Rats were killed by a lethal dose of pentobarbital at 25.5 or 30 hours after infusion. Vessels were perfused via the abdominal aorta with PBS until clear with the jugular vein cut to allow drainage, followed by a 5 minute perfusion with 4% paraformaldehyde in PBS (14). The vessels were then removed from the rat, incubated in paraformaldehyde for one hour, and were stored in PBS until immunostaining. In order to expose the luminal surface for staining, the adventitia was removed, and the vessels were cut lengthwise and pinned open (Minutien pins) onto paraffin wax molds.

Immunohistochemistry for PCNA: The vessels were rinsed 3 times for 10 minutes each in Tris-buffered saline (TBS, 0.05 M Tris-HCl/0.9% NaCl at pH 7.6) and were then treated for 10 minutes with methanol containing 0.6% H_2O_2 to block endogenous peroxidase. The vessels were then rinsed in TBS and incubated in 0.05% trypsin (Gibco, tissue culture grade)/0.1% $CaCl_2$ in TBS at 37°C for 15 minutes. After rinsing in TBS, this was followed by a 30 minute incubation in TBS with 0.1M glycine. Following a TBS rinse, the vessels were incubated for 30 minutes in 2N HCl and rinsed for 5 minutes in 0.1M Borax solution, pH 8.0. After rinsing in TBS, blocking for non-specific binding was done by a 30 minute incubation in Blotto (10% horse serum/0.4% Triton/1% BSA in TBS). PCNA antibody (mouse monoclonal to PC 10, Dako) diluted to 1:4000 in Blotto was applied and the vessels were incubated at 4°C overnight. After rinsing vessels in TBS (3 x 10 minutes), they were incubated for 30 minutes with biotinylated anti-mouse horse immunoglobulin (Vector), followed by peroxidase labelling using avidin-biotin complex (ABC Elite, Vector). TBS with 3,3'-diaminobenzidine (0.037%) (Sigma) and 0.06% H_2O_2 was applied to stain PCNA positive nuclei, followed by counterstaining with hematoxylin.

Häutchen Preparations: In order to observe the endothelium, Häutchen preparations were made (13). Vessels were rinsed in H_2O, dehydrated through alcohol solutions, and incubated in 100% ethanol for 1 hour. After removing the pins, the vessels were covered with 1 drop of 8% (v/v) collodion in 50/50 ether/ethanol. The vessels were then pressed against slides coated with 66% (v/v) collodion in 50/50 ether/ethanol, which had been dried overnight. A 30 minute drying period was followed by a 15 minute incubation in 30% ethanol. The smooth muscle layers were stripped away, followed by application of 5% gelatin in water. The vessels were then pressed against slides previously coated with 5% gelatin which had been allowed to dry, followed by a 15 minute incubation in ice-cold formalin. This was followed by overnight incubation in 10% formalin under pressure (paper clamps). The vessels were then rinsed in H_2O, dehydrated, and mounted under coverslips.

Proliferation Index: The total number of PCNA positive cells adjacent (within 1200 μm) to the denuded region of the artery were counted around the circumference of the vessel. A proliferation index was determined by counting the number of PCNA positive cells within a given field at high power (190 μm) and dividing this number by the total number of cells in that field. The results are expressed as the mean ± S.E.M.
Statistical Analysis: Differences within groups were analyzed by ANOVA (Fisher, PLSD, Statview Software) to assess significance, with $p < 0.05$ regarded as significant.

RESULTS

Vascular endothelial cells are normally quiescent, but after denudation injury by a balloon catheter the endothelial cells near the denuded edge are induced to proliferate in response to the injury (15). We measured the proliferation index at several different times after mechanical denudation injury by an angioplasty catheter (Fig. 1). At the earliest time point (25.5 hours post-injury) the proliferation index for endothelial cells adjacent to the denuded region of the artery was 0.20 ± 0.02, but this quickly increased to 0.79 ± 0.02 at 30 hours. This rapid increase in proliferation reached a plateau by 48 hours, and decreased further by 120 hours. To detect any effect of VEGF infusion on this first wave of endothelial proliferation, it was necessary to choose a time point early in the response to injury. Previous studies indicate little evidence of endothelial proliferation prior to 24 hours aftery injury (15). Therefore we chose to look at the effect of VEGF on proliferation at 25.5 hours (which was more convenient than 24

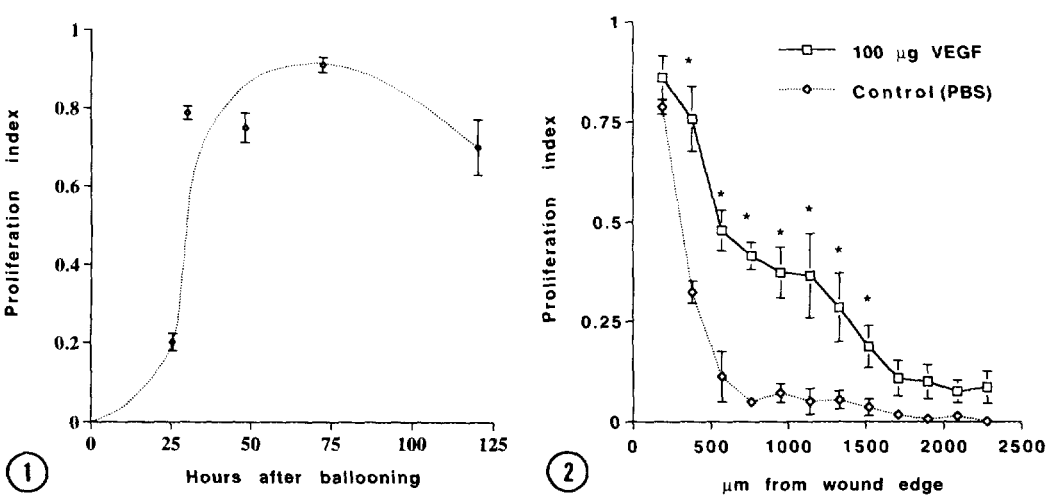

Figure 1. Proliferation index of endothelial cells within 190 µm of the denudation injury, measured at different times after injury. Rats were treated and vessels were stained as described in methods (n = 3 at each time point).

Figure 2. Proliferation index of endothelial cells as a function of the distance from the edge of the denudation injury, after infusion for 3 minutes at the time of injury of either vehicle solution alone or 100 µg VEGF. Rats were treated and arteries examined at 30 hours after injury. (n = 3; *p < 0.05).

hours for our protocol) and 30 hours (as a time-point still within the peak wave of proliferation).

Infusion of 100 μg exogenous VEGF for 3 minutes at the time of denudation injury resulted in significantly greater numbers of endothelial cells in cell cycle (PCNA positive cells) at 30 hours post-infusion. The total number of proliferating endothelial cells within the first 1200 μm from the denuded region increased dramatically by 188% (232 ± 35 to 668 ± 73) after infusion of 100 μg VEGF. The VEGF appeared to induce proliferation of endothelial cells most prominently in the region 190 to 1330 μm away from the denuded edge, as adjacent to the edge (in the first 190 μm), where the index was already high in control animals, infusion of VEGF increased the proliferation index from only 0.79 ± 0.02 to 0.86 ± 0.05 (Fig. 2). These results indicate that VEGF infusion can induce proliferation in endothelial cells within an existing monolayer in the setting of denudation injury, particularly in regions where proliferation is not already maximal. However, the results also indicate that infusion of VEGF at this dose is not sufficient to induce proliferation in all endothelial cells, since cells farther away than 1300 μm from the edge of the injury did not label with PCNA, although these cells were perfused with VEGF.

VEGF infusion also increased endothelial proliferation when assessed at the earlier time. Infusion of 20 μg VEGF at the time of injury, followed by analysis at 25.5 hours resulted in significantly increased proliferation of endothelial cells adjacent to the

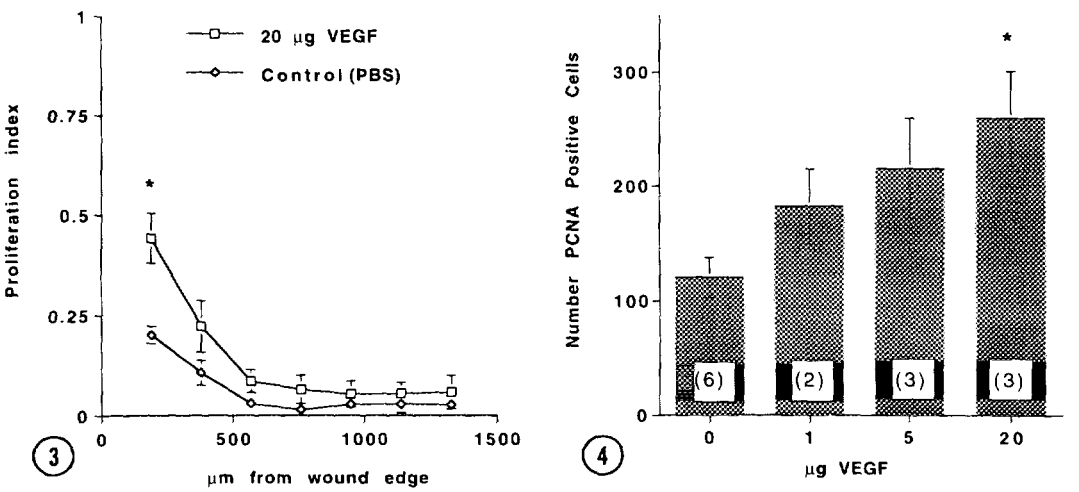

Figure 3. Proliferation index of endothelial cells as a function of the distance from the edge of the denudation injury, after infusion for 3 minutes at the time of injury of either vehicle solution alone or 20 µg VEGF. Rats were treated and arteries examined at 25.5 hours after injury. (VEGF, n = 4; PBS, n = 6; *$p < 0.05$).

Figure 4. Effect of infusion of different amounts of VEGF on the total number of PCNA positive cells 25.5 hours after denudation injury. Rats were treated and vessels were stained as described in methods (n given in parentheses; *$p < 0.05$).

denuded edge, as determined by both proliferation index (0.20 ± 0.02 to 0.44 ± 0.06) (Fig. 3), and total number of proliferating endothelial cells, (120 ± 17 to 259 ± 41) (Fig. 4). Infusion of varying amounts of VEGF at the time of injury, with assessment at 25.5 hours in the manner described for our experiments, increased endothelial proliferation, consistent with a dose dependence of the response.

DISCUSSION

VEGF, an endothelial specific mitogen, is associated with angiogenesis (5,16). Our results indicate that infusion of VEGF for a brief time immediately after denudation of the artery can induce proliferation of normally quiescent endothelial cells. VEGF infusion, when analyzed at 30 hours post-injury, significantly increased the number of proliferating cells in regions of the endothelial monolayer within 1200 μm of the denuded edge. Our results assessed at 25.5 hours post-injury indicate that VEGF infusion also increased the proliferation index at the denuded edge when measured early in the response to vascular injury. From these data we conclude that VEGF can increase endothelial proliferation after vascular injury both in cells within normally proliferative regions and in cells more distant from the wound, which would not normally proliferate at that time under similar conditions.

Mechanical injury induces a wave of endothelial proliferation (15). As described in Fig. 1, the number of endothelial cells near the wound edge involved in proliferation increases rapidly after injury during the first day, but then begins to plateau by two days after injury . A similar time course was described by Schwartz, et al., (15), who found no apparent proliferation, as measured by tritiated thymidine, until 20-24 hours after balloon injury, followed by peak proliferation at 48 hours and decreased proliferation at 96 hours. We chose to assess the effect of VEGF (administered at the time of injury) during the time of rapid increase in proliferative index in order to determine if infused VEGF could actually increase the number of endothelial cells involved in the proliferative response. In addition, the use of an early time point might be more clinically relevant; a rapid regrowth of endothelium may be important to inhibit subsequent SMC proliferation after vascular injury (1).

In addition to demonstrating that VEGF increases proliferation we have shown that the reponse to VEGF increases in a dose-dependent manner. These data suggest that VEGF may play a role in stimulating endothelial proliferation after vascular injury, either alone or through interactions with other growth factors that stimulate endothelial proliferation. Expression of basic fibroblast growth factor (bFGF) is increased in both endothelial cells and SMC after balloon injury of the rat carotid artery (17). Infusion of bFGF also increases endothelial proliferation when administered several weeks after balloon injury (18); it has not been tested whether VEGF also stimulates proliferation this late after denudation. Besides other growth factors directly stimulating endothelial proliferation, it is likely growth factors also induce endothelial proliferation by

modulating VEGF synthesis. For example, synthesis of TGF-β_1, a growth factor which can induce VEGF synthesis in SMC *in vitro*, is increased in the vascular wall in response to balloon injury (19,20,21). Our results are also consistent with a requirement for some other injury-related factor, since endothelial proliferation did not occur throughout the vasculature or even within the entire region directly perfused by VEGF.

The link between vascular wall injury and VEGF synthesis is not clear; nor is the link between VEGF and endothelial proliferation after vascular injury. Our results represent the first description of VEGF infusion increasing endothelial proliferation within the vascular wall after vascular injury. We have demonstrated the effect of VEGF infusion on endothelial proliferation within an existing monolayer, rather than its effect on angiogenesis, which involves capillary sprouting or lengthening (22). Previous studies have focused on the induction or the association of angiogenesis with VEGF. For example, VEGF has been shown to be associated with angiogenesis in tumors, and inhibition of VEGF can decrease tumor growth by inhibiting angiogenesis (5,16,23). VEGF mRNA synthesis is increased in cardiac myocytes after ischemia and in keratinocytes after dermal wounding (6,8). VEGF intraarterial infusion has been shown to increase blood flow and capillary growth after cardiac and hindlimb ischemia (9,24).

Additional studies will be needed to determine whether VEGF synthesis is increased by balloon injury and whether VEGF is required for normal proliferation within the vascular wall. We suggest that increased VEGF may lead to more rapid regeneration of the endothelial layer after injury and suppression of neointimal formation. Our results provide clear evidence that an endothelial specific mitogen can increase endothelial proliferation after balloon injury, and can recruit more endothelial cells to proliferate in response to the denudation injury.

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