Overexpression of VEGF-C Causes Transient Lymphatic Hyperplasia but Not Increased Lymphangiogenesis in Regenerating Skin

Jeremy Goldman, Thomas X. Le, Mihaela Skobe, Melody A. Swartz

Abstract—Vascular endothelial growth factor (VEGF)-C is necessary for lymphangiogenesis and holds potential for lymphangiogenic therapy in diseases lacking adequate lymphatic drainage. However, the ability of VEGF-C to enhance sustainable, functional lymphatic growth in adult tissues remains unclear. To address this, we evaluated VEGF-C overexpression in adult lymphangiogenesis in regenerating skin. We used a model of mouse tail skin regeneration incorporating a suspension of either VEGF-C overexpressing tumor cells, which provide a continuous supplement of excess VEGF-C to the natural regenerating environment for more than 25 days, or otherwise identical control-transfected tumor cells. We found that excess VEGF-C did not enhance the rate of lymphatic endothelial cell (LEC) migration, the density of lymphatic vessels, or the rate of functionality - even though lymphatic hyperplasia was present early on. Furthermore, the hyperplasia disappeared when VEGF-C levels diminished, which occurred after 25 days, rendering the lymphatics indistinguishable from those in control groups. In vitro, we showed that whereas cell-derived VEGF-C could induce chemotaxis of LECs across a membrane (which involves amoeboid-like transmigration), it did not increase LEC chemoinvasion within a 3-dimensional fibrin matrix (which requires proteolytic migration). These results suggest that whereas excess VEGF-C may enhance early LEC proliferation and cause lymphatic vessel hyperplasia, it does not augment the physiological rate of migration or functionality, and by itself cannot sustain any lasting effects on lymphatic size, density, or organization in regenerating adult skin. (Circ Res. 2005;96:1193-1199.)

Key Words: VEGFR-3 ■ chemotaxis ■ lymphatic endothelial cell ■ mouse

Vascular endothelial growth factor (VEGF)-C is necessary for lymphangiogenesis through the binding of VEGFR-3. Although its precise role in lymphangiogenesis remains to be demonstrated, it has recently been shown that excess VEGF-C can cause lymphatic hyperplasia and augment lymphatic growth either in developmental models or in adult tissues after short periods of time following delivery. For example, when overexpressed in the skin of transgenic mice, lymphatic hyperplasia was observed in the tail and increased lymphatic density was seen in the ear. Increased lymphatic density in the ear was also seen following adenoviral VEGF-C overexpression in adult athymic mice and 7 to 8 days after a single delivery of human recombinant VEGF-C whereas VEGF-C gene transfer has been reported to promote new lymphatic vessel growth and reduced edema in both rabbit-ear and mouse-tail skin. Because of these and other findings, VEGF-C therapy holds potential for augmenting the growth of lymphatics in situations requiring increased lymphatic drainage, such as in lymphedema. However, it has recently been shown that VEGF-C overexpressing tumors induce the growth of immature and malfunctional lymphatics in the periphery. Thus, whereas the importance of VEGF-C in lymphangiogenesis is well established, both (1) its specific role in the functional organization of lymphatic structures, and (2) the longer-term fate of newly formed vessels caused by excess VEGF-C as well as their ability to enhance overall lymphatic function, are not known. Until these are clarified, the extent to which excess VEGF-C can augment functional lymphangiogenesis remains unclear.

Here we evaluate the transient response of adult tissue to excess VEGF-C and examine potential limitations in the ability of exogenous VEGF-C to increase lymphangiogenesis in normal adult regeneration. It follows our recent finding that endogenous VEGF-C protein was seen to be upregulated only during the early stages of LEC migration and proliferation in normal adult lymphatic regeneration, but not during the stages of functional organization. We use a cell source of VEGF-C in a mouse model of skin regeneration. In this model, a circumferential section of tail skin is removed and replaced by a collagen matrix kept intact and protected by a...
fine silicone sheath to allow skin regeneration in a relatively scar-free manner. The collagen serves as a scaffold for cell migration and regeneration, and is rapidly remodeled to resemble dermis in terms of extracellular matrix composition and architecture. Because lymphatics are not present in the regenerating region initially, this model uniquely enables us to observe the process of lymphangiogenesis both in terms of function and biology over time, to identify and differentiate new lymphatic growth, and to alter the biochemical environment directly. In this case by suspending VEGF-C overexpressing tumor cells within the collagen before implantation.

We show here that whereas excess cell-derived VEGF-C can cause initial hyperplasia of regenerating lymphatics, it increases neither the rate of LEC migration, the rate of functional lymphangiogenesis, nor the density of regenerating lymphatic vessels compared with controls. Furthermore, the hyperplasia is transient, diminishing after 25 days until the lymphatics organize into a normal functional capillary network. This complements our recent study showing that VEGFR-3 signaling, whereas required for lymphangiogenesis, does not play a role in lymphatic function after lymphatic vessels have developed. To consolidate our finding that excess VEGF-C did not affect LEC migration with previous in vitro data showing a strong chemotactic potential of VEGF-C on LECs, we compare VEGF-C-induced LEC chemotaxis versus chemoinvasion in vitro and show that VEGF-C has no significant effect on the latter. Taken together, our findings, consistent with the potent mitogenic role of VEGF-C in the early stages of lymphangiogenesis, reveal potential limitations in the ability of exogenous VEGF-C to alone augment functional lymphangiogenesis.

Materials and Methods

Lymphangiogenesis Model

A recently developed model of lymphangiogenesis in regenerating adult mouse-tail skin was modified to sustain implanted VEGF-C overexpressing tumor cells into immune-compromised female nu/nu mice (Charles River Labs, Wilmington, Mass). Briefly, a 2-mm wide circumferential band of dermal tissue, in which the lymphatic network in the tail skin is contained, was excised midway up the tail, leaving the underlying bone, muscle, major blood vessels, and tendons intact. This area was covered by a close-fitting, gas permeable silicone sleeve, and type I rat-tail collagen was injected into the region. In some animals, VEGF-C overexpressing, GFP-transfected MDA-MB-435 cells or control transfected counterparts, which did not overexpress VEGF-C (both described elsewhere), were homogeneously suspended in the collagen at 10⁶ cells/mL, before implantation and supplemented with high glucose media (Sigma Aldrich, St. Louis, Mo). The tumor cells provided a continuous source of excess cell-derived VEGF-C in the regenerating region throughout the first 25 days of a 40 day observation period (Figure 1) and the 2 sub-lines provided comparison in our results specifically for VEGF-C effects, as opposed to all other tumor-secreted factors. At least 3 mice were used for each condition at each time point examined, and 46 mice were used in total. Animals were anesthetized with a subcutaneous injection of xylazine (10 mg/kg) and ketamine (87 mg/kg) before surgery and again before microlymphangiography. All protocols were approved by Northwestern University’s Animal Care and Use Committee.

Detection of Functional Lymphatics via Microlymphangiography

To visualize lymph flow patterns both in situ as well as post-fixation in thin sections, a 1% solution of tetramethylrhodamine (TRITC)-conjugated, lysine-fixable dextran of 2×10⁶ Da (Molecular Probes, Eugene, Ore) was injected intradermally into the tail tip where it was taken up and transported by the lymphatics in the proximal direction, revealing the fluid channels and functional lymphatic vessels through which it flowed. The blood vasculature was then perfused with Zamboni’s fixative via the abdominal aorta, revealing the fluid channels and functional lymphatic vessels through which it flowed. The blood vasculature was then perfused with Zamboni’s fixative via the abdominal aorta, which cross-linked the dextran tracer in place for later visualization in thin cryosections, and the tail was snap frozen in liquid nitrogen.

Immunofluorescence and Immunohistochemistry

Tail specimens were cut into 10 μm longitudinal cryosections and incubated with an antibody against the lymphatic-specific hyaluronan receptor LYVE-1 (kind gift from Dr. David Jackson, John Radcliffe Hospital, Oxford, UK) for detection of LECs. A biotinylated antirabbit secondary antibody was used with the LYVE-1 staining (Dako Corp., Carpinteria, Calif) along with alexa fluor 488-conjugated streptavidin (Molecular Probes) for detection; nuclei were labeled with DAPI (Vector Labs, Burlingame, Calif). Lymphatic structures were defined as containing multiple conjoined LECs, and results were compared with controls using a Student’s t test. To detect VEGF-C protein, sections were incubated with a goat

Figure 1. VEGF-C production by tumor cells implanted into regenerating skin. A, VEGF-C protein expression in regenerating skin, as detected by immunostaining at 25 and 40 days, of normal collagen cell-free controls (N), of collagen implanted with a suspension of control-transfected tumor cells (C), and of collagen implanted with a suspension of VEGF-C-transfected tumor cells (V). Dashed white lines indicate location of regenerating region. Distal to proximal direction is shown left to right. Bar length=500 μm. B, Relative VEGF-C protein expression levels in the regenerating zone of each group by quantifying percent area coverage of positive immunostaining. All values are normalized to cell-free controls (N) at 40 days, when VEGF-C was found at baseline levels. Black bars indicate group N, gray bars group C, and white bars group V. *P<0.05.
anti-mouse antibody against VEGF-C (Santa Cruz Biotechnology, Santa Cruz, Calif) with a biotinylated rabbit anti-goat secondary antibody and Vector Red substrate (Vector Labs), and nuclei were counterstained with hematoxylin. VEGF-C labeled images were quantified using a threshold analysis with Axiosvision LE image analysis software.

In Vitro Chemotaxis Assays
Human neonatal dermal microvascular LECs, which had been isolated from human foreskin immunomagnetically using LYVE-1 as described earlier,$^{13}$ were cultured in endothelial basal medium (Cambrex Biosciences, Walkersville, Md) with 20% fetal bovine serum (GIBCO, Carlsbad, Calif) and used at passage 7. VEGF-C-overexpressing tumor cells, the same as those used in the mouse tail skin described above, were cultured in DMEM (Gibco) with 10% serum and 1% penicillin-streptomycin (Sigma). Fibrin was used rather than collagen, specifically to examine proteolytically dependent migration, because cells do not require matrix proteolysis to move through fibrin.$^{16-18}$ First, 8 μm pore size 6.5-mm Transwell polycarbonate membranes (Corning Costar, Cambridge, Mass), coated with 0.2% collagen (Cascade Biologics, Portland, Ore) were turned upside down and 200 000 VEGF-C-overexpressing tumor cells were plated on the bottom surface. After overnight attachment in a 37°C, 5% CO₂ incubator, the wells were turned to their upright position and medium was changed to minimal medium (endothelial basal medium with 2% serum). After 24 hours, medium was again changed to fresh minimal medium and the top surfaces were plated with 5 000 LECs, either alone (to assess proteolytically independent transmigration) or suspended in 3.3 μL fibrin (to assess proteolytically dependent transmigration). After 6 hours, the wells were transferred to 4% paraformaldehyde for 10 minutes and the top surfaces were scraped with a cotton swab. LECs that had migrated to the bottom surface were identified by immunofluorescence for VEGFR-3 (R&D Systems, Minneapolis, Minn) and biotinylated rabbit anti-goat secondary (Dako), and Texas Red-Avidin (Vector Labs) along with DAPI mounting medium (Vector Labs) as a nuclear counterstain. VEGFR-3⁺ cells were counted in five regions for each well. Each condition was repeated at least 5 times and all results were compared using a Student’s t test after confirming normally distributed data.

Results
Abundance of VEGF-C Overexpression in Regenerating Region for More Than 25 Days
To verify the ability of the implanted VEGF-C overexpressing cells to sustainably produce excess VEGF-C in the regenerating region, sections were stained for the presence of VEGF-C protein and compared with control specimens (Figure 1A). At 25 days in regenerating skin supplemented with VEGF-C expressing cells (group V), VEGF-C protein was present in 9-fold excess (Figure 1B) compared with normally regenerating skin (group N; $P=0.0085$) and 4-fold excess to that supplemented with control cells (group C; $P=0.01$). There was also a slight increase in VEGF-C protein in regenerating skin in group C compared with group N at 25 days, but it was not statistically significant ($P=0.08$). Between days 25 and 40, VEGF-C protein was reduced to normal levels ($P=0.35$ between groups N and V) because of the eventual migration of the tumor cells out of the regenerating skin and into the downstream lymphatics.

Tumor Cells, but Not Excess VEGF-C, Enhance Rate of Fluid Channel Formation
To assess the role of tumor cells and excess VEGF-C on the restoration of lymphatic function, microlymphangiography was performed 10, 17, 25, and 40 days after surgery (Figure 2 and Table). In all 3 out of 3 mice for each time point, distinct fluid channels as well as functional continuity of lymphatic capillaries across the regenerating region were seen in groups C and V at 25 days, but not in the normal cell-free controls. At 25 and 40 days, fluid channels were similarly present in all 3 experimental groups. Thus, the implanted tumor cells enhanced the rate of fluid channel formation and functional continuity through the regenerating region as compared with cell-free controls, but equally so for both control-transfected and VEGF-C overexpressing cells. The difference between groups C and N indicates that fluid channel formation was enhanced by the presence of tumor cells, which induce matrix proteolysis and remodeling as well as secrete multiple growth factors including VEGF-A and possibly a small amount of VEGF-C as indicated by Figure 1. The similarity between groups C and V indicates that the excess VEGF-C present in group V, did not lead to increased fluid channeling.

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Relative timing of functional continuity through the regenerating region (e.g. when downstream lymphatic capillaries contain lymph fluid originating in the upstream lymphatics) and capillary regeneration in the regenerating region. Results were consistent for 3/3 mice examined in each time point for each group.
Excess VEGF-C Does Not Affect Rate of Functional Lymphatic Regeneration

To determine the time course of functional lymphatic regeneration, tail sections were stained for the lymphatic specific marker, LYVE-1, and correlated spatially with the dextran fluid tracer (Figure 3). In group N, the regenerating region was nearly devoid of LECs, and fluid tracer was diffuse at 10 days; a few lymphatic structures were present at 17 days with fluid channel formation still in preliminary stages; and finally, lymphatic structures colocalized with lymph fluid tracer at 25 days, suggesting the restoration of functional lymphatic capillaries. In contrast, in both groups with implanted tumor cells (groups C and V), LECs could be seen migrating into the fluid channels from the distal side as early as 10 days, and were partially colocalized with fluid tracer at 17 days, suggesting that at least some lymphatics were functional at day 17, consistent with the findings from microlymphangiography shown in Table 1.

Excess VEGF-C Induces Hyperplasia but Does Not Change Vessel Density or Migration Rate

LEC number, spatial distribution, structure density, and structure size were quantified to determine the effect of excess VEGF-C on lymphatic cell population size and distribution. At 10, 17, and 25 days, but not 40 days, significantly more LECs were present in group V than C (Figure 4A; P=0.01, 0.045, 0.01, and >0.5 respectively). This increase in LEC number was because of lymphatic hyperplasia (Figure 4B) but not increased lymphangiogenesis, because the density of lymphatic structures were not statistically different between groups C and V at any time (Figure 4C; P>0.1 in all groups). Ultimately, the density of lymphatic structures in all 3 groups equalized by 25 days (Figure 4C), despite the much higher VEGF-C expression still seen in Group V at that time (Figure 1).

Next, we examined the relative distribution of LECs along the primary axis of migration to determine differences in migration rate (as opposed to proliferation). It is important to note that LECs migrated primarily from the distal edge of the regenerating region as opposed to the proximal edge, consistent with previous observations that LEC migration occurs in the direction of interstitial fluid flow and lymph flow.9 We evaluated the migration rates by comparing the relative LEC percent distribution in each of 3 regions of the regenerating region (distal or upstream, middle, and proximal or downstream). At 10 days (Figure 5A), the percent distribution of LECs in the distal, central, and proximal region of group N was 79%, 15%, and 5%, whereas it was 42%, 33%, and 26% in group C and 44%, 32%, and 24% in group V. At 17 days (Figure 5B), the distribution in group N was equally distributed in the distal and middle regions (42% and 43%) but low (16%) in the proximal region, whereas LEC distribution was evenly distributed through the regenerating regions in both groups C and V. At 25 and 40 days, LECs were equally distributed throughout the region in all groups. Thus, whereas the cell migration from distal to proximal was clearly slower in the cell-free control group N, there was no significant difference in migration between groups C and V (P>0.5 in all groups).

VEGF-C Induces LEC Chemotaxis but Not Chemoinvasion In Vitro

To put these in vivo findings in perspective with in vitro reports of LEC chemotaxis by VEGF-C, we first confirmed the chemotactic potential of VEGF-C on LECs: in traditional transwell migration assays, we found that of 5 000 LECs...
seeded, 120 ± 43 cells migrated across the 0.8 μm pore size membrane toward VEGF-C-overexpressing cells whereas only 17 ± 8 cells migrated when no tumor cells were present on the bottom (P = 10^−5; Figure 5C). However, whereas this assay demonstrates the chemotactic potential of VEGF-C on LECs, it does not determine whether VEGF-C causes an increase in LEC migration through an extracellular matrix, which would involve stimulating proteolysis by the LECs (i.e., chemoinvasion) and which would be required for in vivo lymphangiogenesis. Thus, we repeated the study with LECs

Figure 4. Size and density of LECs and lymphatic vessels in the regenerating zone. A, Excess VEGF-C transiently increases the density of LECs at 10, 17, and 25 days, but at 40 days, when VEGF-C levels have returned to baseline, LEC density ensuingly returns to normal levels. Black bars indicate group N, gray bars group C, and white bars group V. B, Excess VEGF-C induces hyperplasia of regenerating lymphatics at 10, 17, and 25, but not 40 days. C, Excess VEGF-C does not alter the density of lymphatic structures in regenerating tail skin relative to control tumors. Long-term remodeling of all groups are statistically similar using Student t test.

Figure 5. LEC migration rate unaffected by excess VEGF-C in vivo and in vitro. The regenerating region was divided into thirds along the longitudinal axis of LEC migration (upstream, middle, and downstream locations). Shown is the percent of LECs in each location relative to the total number of LECs in the entire regenerating region, thereby quantifying the distribution of LECs along the migratory axis. A, At 10 days, a comparison of VEGF-C and control tumor cells groups (V and C) revealed a similar distribution of LECs in all 3 locations, demonstrating a similar temporal and spatial distribution of LECs. In contrast, at this time, very few LECs were present in the middle and downstream locations in cell-free control skin (N), indicating a delayed LEC migration (P < 0.05 using Student t test). White, gray, and black bars represent upstream, middle, and downstream locations, respectively. B, At 17 days there is no longer a statistically significant difference between groups, although the mean average distribution in cell-free control skin (N) lags tumor groups V and C. C, In vitro comparison of LEC migration toward VEGF-C overexpressing tumor cells in modified Boyden chambers demonstrates that whereas inducing chemotaxis of LECs across a membrane, VEGF-C does not affect LEC migration through a fibrin gel. Values are normalized to random cell migration (eg, each condition without VEGF-C overexpressing cells). ** P < 0.0005.
suspended in a thin layer (100 μm) of fibrin atop the membrane. The LECs could readily migrate through the fibrin normally in a protease-dependent manner (because the migration was blocked by both aprotinin, a plasmin inhibitor, and GM6001, a broad-range MMP inhibitor; data not shown); of 5000 cells seeded, 32 ± 44 cells crossed the membrane without a chemoattractant (note that fibrin contains a number of promigratory molecules such as fibronectin, thus explaining the higher number and variability than without fibrin). However, unlike the first case, the presence of VEGF-C-secreting tumor cells on the underside of the membrane did not enhance this migration (42 ± 23 migrating cells/5000, P = 0.26; Figure 5C), corroborating our in vivo data and suggesting that VEGF-C may not directly enhance proteolytically dependent LEC invasion through a matrix.

Discussion

We demonstrate that excess cell-derived VEGF-C induces early but transient hyperplasia without altering the rate of LEC migration and eventual lymphatic vessel size, density, organization, and function in regenerating skin. Although it has been reported that excess VEGF-C alone may induce the growth of new functional lymphatic vessels in the adult,5,7,19 our results suggest that these effects may be early and transient, at least with respect to lymphangiogenesis in regenerating tissue. Consistent with this notion, a recent study examining the short-term effects of VEGF-C-overexpressing tumors on lymphangiogenesis showed that the hyperplastic lymphatics induced at the tumor periphery were immature and had poorly developed valves.8

We also found that implantation of tumor cells into regenerating skin enhances the formation of lymph-bearing fluid channels, probably because of the proteases and other growth factors secreted by tumor cells (including VEGF-A and possibly a small amount of VEGF-C) and the high proteolytic activity of these cells, which in turn provides for an early migration of LECs at 10 days. Although VEGF-C has been shown to enhance LEC migration in vitro11,15 and these same cells were shown to increase lymphangiogenesis compared with control-transfected cells when grown as tumors in mice,12 we did not find any evidence of enhanced fluid channel formation or LEC migration in mouse skin supplemented with VEGF-C overexpressing cells relative to skin with otherwise identical control-transfected cells, suggesting that matrix remodeling in the regenerating region may be more important than the excess VEGF-C in early fluid channel formation and LEC migration in vivo. This result is corroborated by our in vitro findings that VEGF-C does not enhance LEC migration through a proteolytically sensitive extracellular matrix, although in vivo it may do so indirectly through the recruitment of proteolytically active macrophages.20,21 Interestingly, we have recently demonstrated that neutralization of VEGFR-3 with antagonistic antibodies does not prevent the formation of fluid channels, but completely inhibits LEC migration and functional lymphatic regeneration in both physiologically normal and excess VEGF-C induced lymphangiogenesis without affecting pre-existing lymphatics.10 Taken together, these findings highlight important differences in the relative roles of VEGF-C versus matrix proteolysis and fluid channel formation in directly inducing LEC migration.

Several aspects of the present model are unique and allow for short- versus long-term evaluation of adult lymphangiogenesis. First, we replace a region of mouse tail dermis with a collagen scaffold, thereby providing a well-defined region for lymphatic growth, allowing us to identify new lymphatic growth and to reproducibly alter the local molecular environment by pre-implanting VEGF-C upregulating tumor cells into the collagen. We note that of all matrix materials we have previously tested in this model, collagen provides the best scaffold for cell migration and matrix remodeling and is quickly remodeled by invading macrophages, neutrophils, fibroblasts, epithelial cells, and blood endothelial cells. Thus, in all the time points observed, the regenerating zone resembles normal dermis in terms of cell and matrix composition (except that hair follicles are absent). Second, because of the simplified unidirectional flow of lymph fluid in the mouse tail and the initiation of LEC migration primarily in the flow direction (described in more detail elsewhere) along with the ability to measure LEC distribution along the longitudinal migratory axis at different times, this model facilitates quantification of LEC migration rate as well as LEC density and structure density. Third, the present model allows for a spatial and temporal identification of lymph fluid and regenerating lymphatic vessels, thereby allowing us to visualize the extent of lymphatic function at different stages of lymphatic regeneration.

VEGF-C overexpressing MDA-MB-435 cells were selected as the VEGF-C delivery vehicle because (1) these cells were previously shown to enhance intra- and peri-tumoral lymphatic density when grown as tumors12 and (2) the cells could be incorporated as a single-cell suspension into a collagen scaffold, which allowed local delivery of excess VEGF-C over long periods of time to a region of regenerating skin. In this manner, we could reproducibly deliver VEGF-C in excess of physiological levels to an otherwise normally regenerating lymphatic network. Our cellular delivery method did not impede normal functional lymphatic regeneration, as verified by implantation of control-transfected tumor cells, although both tumor cells equally increased the rates of lymphatic regeneration.

Our results indicate important differences between the microenvironment of a solid tumor (into which lymphangiogenesis may or may not be induced actively by the tumor) and that of a single-cell suspension of tumor cells within our regenerating skin model (through which lymphangiogenesis is necessary for functional continuity of normal lymphatics). VEGF-C secretion by a solid tumor may induce lymphangiogenesis into a tissue that is otherwise devoid of lymphatics, which is consistent with the requirement of VEGF-C for lymphangiogenesis. However, VEGF-C secretion by those same tumor cells, suspended within a region where lymphangiogenesis would still occur if they were absent, does not augment lymphangiogenesis and has no long-term effects on lymphatic size, density, or function despite early hyperplasia. Thus, our results imply that VEGF-C acts in tandem with other factors to promote physiological lymphangiogenesis.
and that increased VEGF-C does not induce increased lymphangiogenesis under physiological conditions.

In summary, our results demonstrate that excess VEGF-C in an otherwise normal regenerating environment (1) causes early but transient lymphatic hyperplasia in regenerating skin, (2) does not affect LEC migration rate, and (3) does not affect long-term lymphatic vessel density, organization, or function. By differentiating between the short- and long-term effects of excess VEGF-C in adult tissues, our findings suggest that the effects of exogenous VEGF-C on normal lymphatic regeneration are transient and unsustainable.

Acknowledgments

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