Enhanced Capillary Formation Stimulated by a Chimeric Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor-C Silk Domain Fusion Protein

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Abstract—Vascular endothelial growth factor (VEGF)-C and VEGF-D require proteolytic cleavage of the carboxy terminal silk-homology domain for activation. To study the functions of the VEGF-C propeptides, we engineered a chimeric growth factor protein, VEGF-CAC, composed of the amino- and carboxy-terminal propeptides of VEGF-C fused to the receptor-activating core domain of VEGF. Like VEGF-C, VEGF-CAC underwent proteolytic cleavage, and like VEGF, it bound to and activated VEGF receptor-1 and VEGF receptor-2, but not the VEGF-C receptor VEGF receptor-3. VEGF-CAC also bound to neuropilins in a heparin-dependent manner. Strikingly, when VEGF-CAC was expressed via an adeno-virus vector in the ear skin of immunodeficient mice, it proved to be a more potent inducer of capillary angiogenesis than VEGF. The VEGF-CAC–induced vessels differed greatly from those induced by VEGF, as they formed a very dense and fine network of pericyte and basement membrane–covered capillaries that were functional, as shown by lectin perfusion experiments. VEGF-CAC could prove useful in proangiogenic therapies in patients experiencing tissue ischemia. (Circ Res. 2007;100:1460-1467.)

Key Words: proangiogenic therapy • gene therapy • growth factors

The 5 mammalian vascular endothelial growth factor (VEGF) family members identified to date, VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor, are key regulators of physiological and pathological vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability.1,2 VEGF binds to VEGF receptor (VEGFR)-1 and VEGFR-2 and is a potent growth factor for blood vessel formation.1 Furthermore, placenta growth factor, which activates only VEGFR-1, stimulates angiogenesis,3 whereas the angiogenic activity of VEGF-B, which binds to the same receptor, is still under investigation. The biological properties of the VEGFs have been under extensive study, as these molecules are attractive when considering therapeutic angiogenesis, eg, in settings of tissue ischemia.2 VEGF-C and VEGF-D have been shown to activate VEGFR-3 and to induce lymphangiogenesis in various in vivo models.5–9

All members of the VEGF family have a modular domain structure, with the growth factor domain that binds to and activates the receptor, and accessory domains that are mostly involved with binding to the neuropilin receptors and pericellular matrix.1 There are, however, striking differences in the accessory domains. VEGF is expressed as multiple splice variants, including the major forms VEGF121, VEGF165, and VEGF189. These encode proteins that differ in the carboxy terminal (CT) accessory domain. The region of VEGF binding to heparin and heparan-sulfate is coded by exons 6 and 7. Exon 7 also encodes a sequence responsible for binding to neuropilin-1 (NP-1).10 Neuropilins are transmembrane proteins that have a short cytoplasmic domain, and they were first characterized in the nervous system, where they regulate axon growth. NP-1 and NP-2 form complexes with semaphorins, plexins, VEGF family members and VEGF receptors.11 NP-1, which increases the bioactivity of VEGF, is expressed in arteries, whereas NP-2 is mainly expressed in lymphatic vessels and at low levels in veins.12 VEGF165 is secreted, but a large quantity remains bound to the extracellular matrix and cell surface heparan-sulfate. Extracellular matrix–bound VEGF165 can be released by heparin or heparinase, whereas cleavage of VEGF165 by plasmin releases the growth factor domain from the matrix and yields a polypeptide dimer of 34 kDa that can act as a mitogen.13,14 VEGF165 is the most potent angiogenic VEGF isoform, for example, when delivered via an adenovirus to mouse skin.15 In contrast, mouse VEGF120 (corresponding to human VEGF121) lacking the heparin and neuropilin-binding domain cannot

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substitute for the endothelial guidance function of the larger VEGF isoforms.\textsuperscript{16,17} VEGF\textsubscript{165}, on the other hand, binds to neuropilins and to heparin very tightly. Depending on the target tissue, VEGF has also been found to induce hyperplasia of lymphatic vessels.\textsuperscript{15,18–20} VEGF-C and VEGF-D do not bind to heparin.\textsuperscript{5} These factors have CT domains homologous to certain silk proteins, and shorter amino-terminal (NT) propeptides.\textsuperscript{21} Proteolytic cleavage between the growth factor domain and the CT silk homology domain increases the affinity of VEGF-C to VEGFR-3, whereas the NT-cleaved mature form (VEGF-C\textsubscript{ΔNΔC}) can also activate VEGFR-2 in blood vessel endothelial cells, resulting in angiogenic activity.\textsuperscript{5,22–25} Like VEGF, VEGF-C and VEGF-D also interact with NP-1 and NP-2.\textsuperscript{26}

We hypothesized that combining the receptor-activation properties of VEGF with the propeptides of VEGF-C would yield additional information about the biological role of these peptides and possibly result in novel vascular patterns in vivo. In this study, we created a novel chimeric protein, VEGF-CAC, in which the NT propeptide and the CT silk homology domain of VEGF-C flank the VEGF homology domain (VHD), ie, the minimal receptor-binding domain of VEGF, thus replacing the heparin-binding domain. We compared the angiogenic and lymphangiogenic effects of VEGF-CAC with those of VEGF\textsubscript{165}, and with VEGF\textsubscript{109}, which contains only the VHD.

Materials and Methods

Cell Culture

293T (human kidney) and HeLa cells from the American Type Culture Collection (Manassas, Va) were maintained in DMEM (HaartBio, Helsinki, Finland) supplemented with 2 mmol/L L-glutamine (HaartBio), 0.2% penicillin/streptomycin sulfate, and 10% FBS (PromoCell, Heidelberg, Germany). Ba/F3 (murine pro-EpoR) or a VEGFR-2/EpoR were grown in DMEM supplemented with neuropilin–Ig fusion proteins\textsuperscript{26} with or without the addition of 10 μg/mL heparin (GIBCO BRL/Invitrogen). The neuropilin–Ig fusion proteins were produced in 293T cells transiently transfected with the corresponding expression vectors.\textsuperscript{20,32} The complexes were then bound to protein A–sepharose or protein G–sepharose (Amersham Biosciences, Uppsala, Sweden) at 100 μCi/mL for 16 hours. Conditioned medium was collected, cleared by centrifugation, supplemented with 5% BSA and 0.02% Tween-20, and then incubated with anti-human VEGF-1 (AF293NA, R&D Systems, Minneapolis, Minn) or anti-human VEGF-C (AF752, R&D Systems). Alternatively, 1 mL of the conditioned medium was incubated with 200 ng of soluble VEGFR-1-Ig\textsuperscript{23} or VEGFR-2-Ig\textsuperscript{26} or incubated with neuropilin–Ig fusion proteins\textsuperscript{26} with or without the addition of 10 μg/mL heparin (GIBCO BRL/Invitrogen). The neuropilin–Ig fusion proteins were produced in 293T cells transiently transfected with the corresponding expression vectors.\textsuperscript{20,32} The complexes were then bound to protein A–sepharose or protein G–sepharose (Amersham Biosciences), followed by washing 3 times with 0.5% bovine serum albumin and 0.02% Tween-20 in PBS and once with PBS. Proteins were separated by SDS-PAGE under reducing or nonreducing conditions and visualized by autoradiography.

Pulse–Chase Analysis

Transfected Ba/F3 cells were metabolically labeled for 30 minutes and then chased in nonradioactive medium for different time periods. The conditioned media were supplemented with 5% BSA and 0.02% Tween-20, and recombinant VEGF proteins were immunoprecipitated. Antigen–antibody complexes were analyzed under reducing conditions.

Bioassay for Growth Factor–Mediated Cell Survival

HeLa cells (10\textsuperscript{4}) were seeded on 10-cm plates and transduced with AdVEGF-CAC, AdVEGF\textsubscript{109}, AdVEGF\textsubscript{165}, or AdLacZ. After 24 hours, the transduced cells were serum starved for 16 hours; after which time, medium was collected, centrifuged at 2500 rpm for 5 minutes and stored at 4°C. To compare expression levels of the different constructs, conditioned medium was mixed with Laemmli sample buffer, heated for 10 minutes at 95°C and separated in 12% SDS-PAGE gels (Ready-Gel, Bio-Rad). Proteins were transferred to a nitrocellulose membrane, blocked with 5% BSA, and incubated with anti-VEGF. Biotinylated rabbit anti-goat and streptavidin–horseradish peroxidase were used as secondary antibodies. Bound antibodies were visualized using the Femto ECL reagent (Amer sham) according to the instructions of the manufacturer.

Ba/F3 cells expressing the VEGFR-1/EpoR\textsuperscript{23} or VEGFR-2/EpoR chimeric receptor\textsuperscript{32} were seeded into 96-well plates at the density of 20 000 cells per well in triplicates and supplied with different dilutions of conditioned medium from HeLa cells. Cell viability was quantified after 48 hours by a colorimetric assay. Briefly, 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, Mo) was added into each well and incubated for 2 hours at 37°C. The reaction was terminated by adding lysis buffer (10% SDS/10 mmol/L HCl), and the resulting formazan products were solubilized overnight in a humid atmosphere. The absorbance at 540 nm was measured using a Multiscan microtiter plate reader (Thermo Labsystems, Milford, Mass).

In Vivo Analysis of Adenoviral Vectors

Approximately 2×10\textsuperscript{5} pfu of AdVEGF-CAC, AdVEGF\textsubscript{109}, AdVEGF\textsubscript{165}, or AdLacZ were injected subcutaneously into the ears of NMRI nu/nu mice (Taconic Europe, Mollegaard, Denmark). Two weeks after adenoviral gene transduction, the mice were anesthetized and then perfused with 1% paraformaldehyde for 2 to 3 minutes. The conditioned cells and immunoprecipitation from conditioned media, followed by SDS-PAGE and autoradiography.
ears were collected, immersed in 4% paraformaldehyde for 2 hours, and dissected for whole-mount staining. Alternatively, the ears were embedded in OCT medium (TissueTek, Sakura Finetek, Zoetemoude, The Netherlands), frozen, and cut into 6-μm sections. The tissues were blocked with 5% normal goat or donkey serum in 0.3% Triton X-100 (Fluka Biochemika, Steinheim, Switzerland) in PBS. For staining of blood vessels in the ear, tissues were incubated overnight with a monoclonal hamster anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 antibody (clone 2H8, Chemicon, Temecula, Calif). Perfused blood vessels were visualized by injecting the mice intravenously with 1 mg of fluorescein isothiocyanate (FITC)-conjugated *Lycopersicon esculentum* lectin (LE-lectin) (VectorLabs, Burlingame, Calif) in 200 μL of PBS, followed by perfusion fixation with 1% paraformaldehyde. For detection of smooth muscle cells, staining was performed with antibodies for smooth muscle actin (clone 1A4, C6198, Sigma). Basement membranes of blood vessels were stained with monoclonal rat antibodies against nidogen/entactin (Chemicon), and pericytes with monoclonal rabbit antibodies against neuron-glial protein 2 (NG2, Chemicon), whereas lymphatic vessels were visualized with rabbit antisera against the lymphatic endothelial–specific hyaluronan receptor LYEVE-1.33 Samples were incubated with the primary antibodies, followed by overnight incubation with appropriate fluorophore-conjugated secondary antibodies (Alexa 488 or Alexa594 [Molecular Probes, Eugene, Ore] or FITC [Jackson ImmunoResearch, Bar Harbor, Me]). Fluorescently labeled samples were mounted with Vectashield (VectorLabs) and analyzed with a compound fluorescent microscope (Zeiss 2, Carl Zeiss, Göttingen, Germany; objective ×10 with numerical aperture [NA] 0.30) or a confocal microscope (Zeiss LSM 510; objectives ×40 with NA 1.3 and ×63 with NA 1.4) by using multichannel scanning in frame mode. Three-dimensional projections were constructed digitally from confocal z-stacks.

Semimembranous muscles of rabbit hindlimbs were adenovirally transduced with AdVEGF-CAC, AdVEGF109, AdVEGF165, and Ad-LacZ and analyzed as described previously.34 All experimental procedures involving laboratory animals were approved by the Provincial State Office of Southern Finland (mice) and Eastern Finland (rabbits).

**Quantitative Analysis**

ImagePro Plus version 6.0 software (Media Cybernetics Inc, Silver Spring, Md) was used to quantify the LE-lectin–perfused vessel area and the area of PECAM-1–positive endothelium. Vessels surrounded by vascular mural cells were counted as PECAM-1–positive vessels that were in contact with NG2–positive pericytes, or smooth muscle actin–positive smooth muscle cells. The cross-sectional area of capillaries in rabbit semimembranosus muscle was counted from actin–positive smooth muscle cells. The cross-sectional surface area and the area of PECAM-1–positive endothelium. Vessels surrounded by vascular mural cells were counted as PECAM-1–positive vessels that were in contact with NG2–positive pericytes, or smooth muscle actin–positive smooth muscle cells. The cross-sectional area of capillaries in rabbit semimembranosus muscle was counted from actin–positive smooth muscle cells.

**Results**

**Proteolytic Processing and Receptor Binding and Activation Properties of the VEGF-CAC Chimera**

To analyze the functions of the VEGF-C propeptides, we cloned the VEGF-CAC construct comprising the minimal receptor-binding domain (VHD or VEGF109) of VEGF between the NT and CT propeptides of VEGF-C (Figure 1A). For comparison, we used VEGF165 and VEGF109 as controls throughout the study. The factors produced by the constructs were analyzed by binding of the 35S-labeled conditioned medium from transiently transfected 293T cells to the soluble extracellular domains of the VEGF receptors. As shown in Figure 1B, the VEGF109 polypeptide protein bound to VEGFR-1 and VEGFR-2 but not significantly to the neuropilins, whereas the VEGF-CAC protein bound also to the neuropilins. Interestingly, the VEGF-C propeptides did not inhibit VEGF-CAC binding to VEGFR-1 or VEGFR-2, although they are known to severely reduce VEGF-C and VEGF-D binding to VEGFR-2. Heparin greatly increased the binding of VEGF-CAC to both neuropilins; interaction especially with NP-2 was almost undetectable in the absence of heparin (Figure 1B). In nonreducing conditions, the full-length VEGF-CAC migrated as an ~100-kDa polypeptide band (Figure 1C), indicating that it formed disulfide-linked dimers.

**VEGF-CAC protein processing was analyzed by pulse-chase labeling and immunoprecipitation with anti-VEGF-C antibodies.** VEGF-CAC and VEGF-C were secreted as 66- and 68-kDa polypeptides, respectively. Both were processed in a similar manner into doublets of approximately 29 and 31 kDa containing the 31-kDa CT part and the 29-kDa NT part, together with the VHD. Gradually, small amounts of the
20-kDa mature forms containing the VHD were generated from both polypeptides (Figure 1D). The unprocessed forms of neither protein were detected after 24 hours, indicating that they were effectively processed.

Both AdVEG-CAC and AdVEGF109 bound to VEGFR-1 and VEGFR-2 but not to VEGFR-3 (Figure 2A). The ability of the adenovirally produced factors to dimerize their receptors was tested in the Ba/F3-VEGFR-1/EpoR and Ba/F3-VEGFR-2/EpoR cells. The growth and survival of these cells depends on the dimerization of the respective receptors and activation of the intracellular domain of EpoR. Expression levels of AdVEG-CAC and AdVEGF109 in the conditioned media of adenovirus-transduced HeLa cells were approximately equal (Figure 2B), and both media activated VEGFR-1 and VEGFR-2 in roughly similar dilutions, but they did not activate VEGFR-3 (Figure 2C and data not shown). These data indicated that both factors were capable of receptor binding and activation.

VEG-CAC Induces Angiogenesis and Lymphatic Vessel Dilation In Vivo

To assay the biological activity of VEG-CAC in vivo, AdVEG-CAC was injected into rabbit hindlimb skeletal muscle and into the ears of nude mice. AdVEG-CAC induced a 37.5-fold increase and AdVEGF109 a 50.7-fold increase in cross-sectional capillary surface area compared with AdLacZ controls (P<0.01), whereas the increase in AdVEGF165-transduced muscle was 20.1-fold (P<0.01), which was significantly less when compared with AdVEG-CAC or AdVEGF109 (P<0.05) (Figure 3A through 3D). Two weeks after gene transduction, pronounced swelling and erythema was observed in mouse ears transduced with AdVEG-CAC, AdVEGF109, and AdVEGF165 in a decreasing order, whereas no such effects were seen in the AdLacZ-treated ears (data not shown). Whole-mount immunofluorescent staining of the AdVEG-CAC–transduced ears revealed an extensive hyperplastic network of PECAM-1–positive capillaries and enlargement of the lymphatic capillaries. The effects induced by VEGF109 and VEGF165 were less extensive; in particular, such extensive fine network of very small new capillaries was not seen in the VEGF109- or VEGF165-transduced ears (Figure 3E through 3H). Circumferential hyperplasia of the larger arteries and veins of the ear was also observed in the ears treated with any of the 3 growth factors (data not shown).
The AdVEGF-CAC–Induced Capillaries Are Coated by Basement Membrane and Pericytes

Whole-mount preparations of AdVEGF-CAC–transduced ears stained with antibodies to nidogen/entactin and PECAM-1 showed a prominent increase in thin basement membrane tubes that contained endothelial cells (Figure 4A through 4C), when compared with the ears treated with the other 2 factors (Figure 4D through 4I). The major difference between AdVEGF-CAC– and AdVEGF109- or AdVEGF165–transduced vessels was the striking pattern that was a homogenous network in the former and an uneven tree-like hierarchical branching, containing new sprouts in the latter 2 sets of vessels.

The AdVEGF-CAC–induced angiogenesis was also accompanied by vessel investment by smooth muscle cells. We found that 71.0% (SEM, 14.8%) of the blood vessels had smooth muscle cell coverage in VEGF-CAC–transduced ears, whereas fewer such vessels were observed in VEGF165–transduced (33.3 ± 13.0%), VEGF109–transduced (40.8 ± 14.7%) ears (P < 0.05). The proportion of vessels encircled by pericytes in VEGF-CAC ears was similar to that observed in ears after LacZ transduction (62.2 ± 5.8%), reflecting a normal degree of vessel stabilization in VEGF-CAC–transduced ears.

The AdVEGF-CAC–Induced Vessels Are Functional

To visualize perfused blood vessels, the mice were injected intravenously with fluorescent L esculentum (tomato) lectin, which binds to N-acetyl-D-glycosaminoglycan in blood vascular endothelial cells. Mouse ears transduced with AdVEGF-CAC showed a dramatic increase in the number of lectin-positive vessels when compared with the 2 other VEGFs, although some of the PECAM-1–positive endothelium remained lectin negative in all cases (Figure 5). Area-density quantification of LE-lectin and PECAM-1–positive vessels from the different ear sections is shown in Figure 5M and 5N, with the ratio of lectin-perfused vessels to PECAM-1–stained vessels in each case in Figure 5O.

Discussion

We report here the generation and biological characterization of VEGF-CAC, a chimeric VEGF comprising the receptor-activating domain of VEGF flanked by the propeptides of

![Figure 4.](image-url) The VEGF-CAC–induced capillaries are coated with basement membrane proteins and pericytes. A through L, Immunofluorescent staining for PECAM-1 (green) and the basement membrane nidogen/entactin (red) in ears transduced with the indicated adenoviruses. Arrows indicate vessels positive for PECAM-1 and nidogen/entactin. M through T, Staining for PECAM-1 (green) and smooth muscle actin (SMA) (red) or neuron–glial protein 2 (NG2) (red) shows increased pericycle coating of the vessels induced by AdVEGF-CAC (M and N), when compared with controls (O through T). The red signal in the cartilage in (T) is tissue autofluorescence.
VEGF-C. VEGF-CAC is secreted and processed in a manner similar to VEGF-C, and it induces angiogenesis in a pattern distinct from VEGF.

In vitro, VEGF-CAC was a potent inducer of the proliferation/survival of BaF3 cells expressing chimeric VEGF receptors, whereas in vivo overexpression of the factor led to robust angiogenesis that was shown to exceed even the angiogenic activity of VEGF165. This increase in biological activity may be attributable to the greater solubility of the immature VEGF-CAC when compared with VEGF165, as VEGF-CAC does not have a heparin-binding domain. The architecture of the vessels formed in response to adenoviral overexpression of VEGF-CAC differed from that induced by VEGF165. VEGF-CAC induced a more homogenous network of perfused, pericyte- and basal lamina–coated capillaries, instead of a more hierarchical structure of vessels of differing sizes in a treelike branching pattern generated by VEGF165.

VEGF165 stimulates angiogenesis through capillary sprouting by inducing concentration-gradient-guided migration of endothelial cells. This coordinated action is apparently attributable to the intermediate heparin-binding properties of VEGF165, when compared with VEGF121 and VEGF189. VEGF bioavailability is regulated extracellularly by matrix metalloproteinases through intramolecular processing and subsequent release from the matrix. An elegant study by Lee et al showed that a mutant matrix metalloproteinase–resistant VEGF supported extensive growth of thin vessels with multiple and frequent branch points. According to their findings, matrix-bound VEGF and nontethered VEGF provided different signaling outcomes and vascular patterning.

It is possible that VEGF-CAC promotes extensive development of thin-capillary-sized vessels because most of this growth factor is only partially processed, with a retention of the silk homology domain that, like silk, could have a structural function in the pericellular matrix. Interestingly, the newly formed capillaries were abundantly covered with pericytes, which are known to stabilize blood vessels via angiopoietin-1 and sphingosine-1–phosphate signals, indicating that the vessels were more functional, stable and mature than those generated by VEGF165 or VEGF109.

Figure 5. AdVEGF-CAC induces the formation of perfused blood vessels. PECAM-1 (red) staining of ear sections from mice injected with the indicated adenoviruses and perfused with FITC-conjugated LE-lectin (green). M through O, Digital area density quantification of LE-lectin–positive blood vessels and PECAM-1–positive endothelium. Bars represent mean values±SD (n=3). Note that the area of lectin-positive vessels shows a 5.1-fold increase in perfused vessels in AdVEGF-CAC–transduced ears, compared with the LacZ control, and a 2.0-fold increase compared with VEGF109. The area of PECAM-1–positive vessels in AdVEGF-CAC–transduced ears was 3.2 greater than in AdLacZ–transduced ears and 2.1-fold greater than in VEGF109–transduced ears. O, The ratio of LE-lectin–lectin-positive blood vessels to PECAM-1–positive endothelium. *P<0.05 compared with LacZ, **P<0.05 compared with all other groups.

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In addition to its angiogenic activity, VEGF-CAC was shown to induce circumferential dilation of cutaneous lymphatic vessels. Consistent with our results, overexpression of VEGF has been shown to induce large, hyperplastic lymphatic vessels.\textsuperscript{15,18,19} These signals may be mediated via VEGFR-2, which is also expressed in lymphatic endothelial cells.\textsuperscript{6,20} The effects of VEGF-CAC on lymphatic vessels could be at least partly attributable to increased fluid accumulation as a result of VEGFR-stimulated blood vessel leakiness.

Both VEGF and VEGF-C are well-characterized growth factors, and a wealth of knowledge about their biological functions exists.\textsuperscript{1} However, knowledge about the biological function of VEGF-C propeptides has remained scarce, whereas the role of the heparin-binding domains of VEGF has been abundantly studied.\textsuperscript{2} Our approach to study VEGF-C propeptides by swapping domains between growth factors has led previously to generation of novel and particularly potent growth factor variants. For example, replacement of the NT oligomerization domain of angiopoietin-1 with the N terminus of angiopoietin-2 yielded a more soluble and active growth factor, designated Ang1*.\textsuperscript{41} Furthermore, linkage of the heparin-binding domain of VEGF\textsubscript{165} to VEGF-E resulted in a heparin-binding form of VEGF-E (hbVEGF-E), which was able to stimulate proliferation and sprouting of endothelial cells.\textsuperscript{42} In a binding competition assay, hbVEGF-E was more potent than VEGF-E, which may have been caused by binding to the NP-1 coreceptor.\textsuperscript{42} As VEGF-CAC proved to potently promote the formation of new capillaries with a distinct morphology, we envision significant potential for such domain-swap combinations in biotechnology applications in the future.

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