Up-regulation of VEGF expression by NGF that enhances reparative angiogenesis during thymic regeneration in adult rat

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Abstract

Angiogenesis is important for adult tissue regeneration as well as normal development. Vascular endothelial growth factor (VEGF) is a unique potent angiogenic factor, and plays an essential role in regulating angiogenesis during embryonic development, normal tissue growth, and tissue regeneration. Recent evidence shows that nerve growth factor (NGF) also plays a role as an angiogenic regulator as well as a well-known neurotrophic factor. The aim of this study was to investigate whether thymus regeneration accompanies reparative angiogenesis and also to evaluate whether the thymic expression of VEGF is regulated by NGF in vivo and in vitro. Here, we show that high VEGF mRNA and protein levels are concomitant with reparative angiogenesis that occurs dramatically during regeneration following acute involution induced by cyclophosphamide (CY) in the rat thymus. Fluorescent thymus angiography using FITC-dextran showed that thymic regeneration is associated with a much denser capillary network compared with normal control thymus. Furthermore, the expressions of NGF and TrkA were highly increased during thymic regeneration. We also show that NGF mediates thymic epithelial induction of VEGF expression in vitro and in vivo. Taken together, our results suggest that NGF-mediated VEGF up-regulation in thymic epithelial cells may contribute to reparative angiogenesis during thymic regeneration in adult.

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1. Introduction

Angiogenesis, the formation of new capillary blood vessels from existent microvessels [1], is critical for normal embryonic development and also contributes many pathological states including tumor growth [2,3]. In addition, angiogenesis is essential for tissue regeneration in adults [4,5]. In the physiological condition, the activity of inducers and inhibitors of angiogenesis maintains it in balance [2,6].

Vascular endothelial growth factor (VEGF), a highly specific mitogen for vascular endothelial cells, regulates vasculogenesis, angiogenesis, and vascular maintenance during embryogenesis and in adults [3,7–9]. VEGF binds to three receptor tyrosine kinases (RTKs), known as VEGF receptor-1 (Flt-1), -2 (KDR) and -3 (Flt-4), as well as to the neuropilin-1 and neuropilin-2 coreceptors [10]. The binding of VEGF to the receptors induces receptor dimerization and activation, resulting in the proliferation and growth of endothelial cells [11]. VEGF expression is up-regulated by hypoxia [12] or by different cytokines and growth factors even in normoxia [13–15]. Recent evidence shows that nerve growth factor (NGF) is able to induce the expression of VEGF in neuronal tissue [16] and in tumor [17]. NGF, a well-known neurotrophin in the nervous system [18], has been recently described as an ‘angiogenic’ molecule
[19], either directly, via promoting endothelial cell proliferation and migration [20,21], or indirectly by increasing VEGF gene expression [22,23]. NGF forms covalent homodimers that activate two types of receptor, the TrkA (tropomyosin-receptor kinase A) and p75NTR receptors [24,25]. Our recent studies have demonstrated that the expressions of NGF and TrkA receptor were up-regulated in the thymic epithelial cells during rat thymic regeneration following cyclophosphamide-induced acute invasion [26,27]. However, the role of NGF/TrkA signaling in the process of thymic regeneration remains to be established.

Thymus has a central role in the immune system and harbors an organ-typical dense network of branching and anastomosing blood vessels [28,29]. It has been well documented that acute thymic involution, induced in experimental animals, is followed by intensive regeneration after the removal of the causative stimuli [30]. Although recent studies regarding the roles of angiogenesis in stimulating adult tissue regeneration have been published [4,5,31], they do not much address the molecular basis of the reparative angiogenesis during adult tissue repair/regeneration, especially in the thymic regeneration.

The purpose of this study was to examine whether reparative angiogenesis is accompanied by adult thymic regeneration and to investigate the expression patterns of VEGF and its regulation by NGF signaling in thymic epithelial cells. We examined blood vessel network and VEGF expression in regenerating thymus. We next studied the effect of NGF on in vivo VEGF expression and thymic vascularization, and its effect on in vitro VEGF expression.

2. Materials and methods

2.1. Reagents and antibodies

Rabbit polyclonal anti-human VEGF and mouse monoclonal anti-human VEGF antibodies were purchased from Santa Cruz Biotechnology and Upstate, respectively. Mouse monoclonal anti-rat CD31 (PECAM-1) and rabbit polyclonal anti-human VEGF antibodies were purchased from BD Pharmingen and Chemicon, respectively. Goat polyclonal anti-human TrkA and rabbit polyclonal anti-human TrkA antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-human Cytokeratin antibody was purchased from BD Pharmingen and Chemicon, respectively. Goat polyclonal anti-human TrkA and rabbit polyclonal anti-human TrkA antibodies were purchased from Santa Cruz Biotechnology and Chemicon, respectively. Mouse monoclonal anti-rat CD31 (PECAM-1) antibody, diluted 1:100 (Santa Cruz), or with a mouse monoclonal anti-CD31 (PECAM-1) antibody, diluted 1:200 (BD bioscience). Following incubation with the primary antibody, the sections were washed three times for 5 min with PBS and incubated for 1 h at room temperature with goat anti-rabbit biotinylated antibody diluted 1:100 (Jackson ImmunoResearch Laboratories) or with donkey anti-mouse biotinylated antibody diluted 1:100 (Jackson ImmunoResearch Laboratories). They were then rinsed in PBS and incubated for 60 min at room temperature with an ABC reagent (Vectorstain Elite Kit; Vector Laboratories) according to the manufacturer’s instructions. The sections were developed in 0.05% 3, 3'-diaminobenzidine and 0.003% H2O2 medium under microscopic control at room temperature to visualize peroxidase activity. Light microscopy slides were observed and photographed using an Olympus BX50 microscope. All photomicrographs were taken with an Olympus C-3030 digital camera.

2.2. Experimental acute thymic involution and regeneration model

Adult male specific pathogen-free Sprague–Dawley rats were purchased from Dae Han Bio Link (Seoul, Korea). All rats were housed three to four per cage and maintained under a 12-h light/dark cycle at 24 °C in a specific pathogen-free and humidity-controlled facility. Animals were provided with standard sterile food and water ad libitum, and were allowed to adjust to their environment for 1 week. Animals were used at 8–10 weeks of age, by being given a single intraperitoneal dose of cyclophosphamide (150 mg/kg body weight, Sigma) in normal saline [26,30], and were sacrificed in group of four at 3, 7 and 14 days after injection. Rats given the same amount of normal saline were used as controls. Three independent experiments were performed, with 16 animals in each experiment (four animals in each group). Animal care and all experimental procedures were conducted in accordance with the “Guide for Animal Experiments” edited by the Korean Academy of Medical Sciences.

2.3. Angiography

To visualize blood vessels by whole-mount fluorescence microscopy, the rats were injected i.v. with 2 x 10^6 molecular weight FITC-dextran (50 mg/ml, Sigma) [32]. Ten minutes after the injection, rats were sacrificed. For cryosections, the thymus was removed and rapidly frozen in isopentane cooled with liquid nitrogen. Frozen sections (30 μm thick) were cut on a Reichert cryostat and placed on 3-aminopropyltriethoxysilane-coated slides. The samples were examined under a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany).

2.4. Immunohistochemistry

After cryosections, immunostaining was performed by using the streptavidin–biotin complex (ABC) method. In brief, the sections were incubated for 20 min in a solution of phosphate-buffered saline (PBS) containing 0.3% H2O2 to eliminate endogenous peroxidases. After washing in PBS, the sections were incubated with 3% BSA (BSA, Sigma). The excess solution was shaken off and the sections were incubated for 16–18 h at 4 °C with a rabbit polyclonal anti-VEGF antibody, diluted 1:100 (Santa Cruz), or with a mouse monoclonal anti-CD31 (PECAM-1) antibody, diluted 1:200 (BD bioscience). Following incubation with the primary antibody, the sections were washed three times for 5 min with PBS and incubated for 1 h at room temperature with goat anti-rabbit biotinylated antibody diluted 1:100 (Jackson ImmunoResearch Laboratories) or with donkey anti-mouse biotinylated antibody diluted 1:100 (Jackson ImmunoResearch Laboratories). They were then rinsed in PBS and incubated for 60 min at room temperature with an ABC reagent ( Vectastain Elite Kit; Vector Laboratories) according to the manufacturer’s instructions. The sections were developed in 0.05% 3, 3'-diaminobenzidine and 0.003% H2O2 medium under microscopic control at room temperature to visualize peroxidase activity. All photomicrographs were taken with an Olympus BX50 microscope. All photomicrographs were taken with an Olympus C-3030 digital camera.

2.5. Immunocytochemistry

Cells cultured on a coverglass were fixed in 10% paraformaldehyde (in phosphate buffered saline) for 10 min, blocked with 0.3% Triton X-100/10% fetal bovine serum/PBS for 1 h, and then labeled with appropriate primary antibodies. After overnight incubation at 4 °C, the cells were washed with cold-PBS three times and incubated with FITC-conjugated secondary antibodies for 1 h at RT. Coverslips were mounted in Vectastain containing DAPI (Vector Laboratories). Cells were analyzed using fluorescent microscope (Axioskop, Zeiss, Oberkochen, Germany).

2.6. Western blot

Samples (30 μg total protein/lane) were dissolved with loading buffer (0.1 M Tris–HCl buffer pH 6.8, containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12% SDS-PAGE, and electrophotographically transferred to nitrocellulose membrane (Amersham-Pharmacia Biotech) by semidy transfer (Bio-Rad). The membranes were incubated for 40 min at room temperature with blocking buffer (10% skim milk, 10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20). Membranes were washed three times for 10 min each at room temperature in TTBS (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) followed by an incubation for 1 h at room temperature with rabbit anti-VEGF or with mouse anti-<i>α</i>-actin. Membranes were washed three times for 10 min each at room temperature in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody. The blots were developed with ECL (Amersham Pharmacia Biotech) as the chromophore.
2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from each sample using Trizol RNA Extraction Reagent (Bio Rad) following the manufacturer’s protocol. First-strand cDNA synthesis was carried out on 2 μg of total RNA with a reverse transcription kit (Invitrogen). One-tenth of the reaction mixture was used as template for PCR amplification. The PCR reaction condition and the sequences of primers are available upon request. After the PCR, the amplified products were analyzed by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining under UV light illumination. Band intensities of the PCR products were measured using an image analysis program (MetaMorph, Universal Imaging Corporation). Data were expressed as ratios of each mRNA normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA which was used for internal control.

2.8. Cell line and culture

Thymic subcapsular/cortex epithelial cell (427.1; SNEC) line was kind gift of Dr. Barbara B. Knowles (The Jackson Laboratory). The cells were cultured in Dulbecco’s modified Eagle medium (Gibco-BRL) supplemented with 10% Fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO2 in 95% air. Compound K252a (100 nM, Sigma), a tyrosine-kinase inhibitor, was used to study involvement of the TrkA receptor. Cells were pre-treated with K252a or its solvent for 1 h before NGF (Allomon) treatment.

2.9. In vivo NGF injection

Mice were used at 8–10 weeks of age, by being given a single intraperitoneal dose of cyclophosphamide (450 mg/kg body weight, Sigma) in normal saline. Six
hours after injection, one group was intraperitoneally injected with 10 μg of NGF (Allomon). Another control group received intraperitoneally injection of saline solution. Mice were sacrificed in group of four at 2 and 5 days after NGF injection, respectively. For cryosections, the thymus was removed and rapidly frozen in isopentane cooled with liquid nitrogen. Frozen sections (5 μm thick) were cut on a Reichert cryostat and placed on 3-aminopropyltriethoxysilane-coated slides.

3. Results

3.1. Thymic regeneration accompanies reparative angiogenesis

Thymus regenerates to its normal mass within 14 days after acute involution induced by cyclophosphamide (CY) in the rat (Fig. 1A). To investigate whether the thymic regeneration accompanies reparative angiogenesis, fluorescein isothiocyanate-dextran (FITC-dextran) was used for angiography. The FITC-dextran angiography revealed that thymic blood vessel density was significantly elevated at 3 days after CY treatment, and levels peaked at this time point and then declined by 14 days (Fig. 1B), an observation confirmed by immunostaining for PECAM-1 (Fig. 1C). Similar results were obtained by immunostaining for von-Willebrand factor (data not shown).

3.2. Upregulation of VEGF, TrkA and NGF expressions during thymic regeneration

A number of proteins have been reported to be induced during angiogenesis [1,2]. These include VEGF, a well-known potent angiogenic factor [7,8]. Since angiogenesis was dramatically induced during thymic regeneration (Fig. 1), we examined the expression pattern of VEGF in regenerating thymus. Immunostaining for VEGF protein revealed that the levels of VEGF protein were highest at 3 days after CY treatment (Fig. 2A: c and d) and then decreased gradually to basal level by 14 days (Fig. 2A: e to h). To assess whether the changes of VEGF protein levels in the regenerating thymus were due to a local production of the VEGF, we measured the levels of VEGF
mRNA by RT-PCR analysis. As shown in Fig. 2B, the levels of VEGF mRNAs were markedly increased at 3 days after CY treatment by ∼7.3-fold over the normal control. Furthermore, consistent with previous data [26,27], the increase in TrkA mRNA level was apparent throughout the time course, with peak expression at 3 days (∼2-fold elevation over control).

Fig. 2. Up-regulation of VEGF, TrkA and NGF expressions during thymic regeneration. (A) Immunohistochemical localization of VEGF in the cortex (a, c, e, g) and medulla (b, d, f, h) of the normal thymus (a–b) and regenerating thymus at 3 (c–d), 7 (e–f) and 14 (g–h) days after CY treatment. Magnification is 200×. Inner panel (100X). (B) RT-PCR analysis showing mRNA levels of VEGF, TrkA, NGF, and FoxN1 in the normal thymus (N) and regenerating thymus at 3, 7, and 14 days after CY treatment (left). Relative VEGF, TrkA, and NGF mRNA levels normalized with GAPDH mRNA (right). The density of each band in each lane from three independent experiments was quantified by scanning densitometry and then expressed as mean±SD.
during thymic regeneration (Fig. 2B). Also the expression of NGF, which acts as a TrkA ligand, was elevated in the regenerating thymus at 3 days after CY treatment and then gradually declined by 14 days (Fig. 2B). To investigate the possibility that the upregulation of VEGF, TrkA, and NGF may be due to thymus composition (e.g. more TEC and less thymocytes), we checked the expression pattern of FoxN1, which is specifically expressed in thymic epithelial cells in thymus [33], and found that the levels of FoxN1 mRNA was unchanged during thymic regeneration (Fig. 2B). Therefore, the upregulation of VEGF, TrkA and NGF mRNA expression is likely due to the increased expression in thymic epithelial cells (TEC) rather than changes in thymic cellular composition.

3.3. Co-localization of VEGF in TrkA-positive thymic epithelial cells (TECs)

We next examined what types of cells are responsible for the VEGF expression. Double-immunohistochemistry for VEGF and cytokeratin (CK) revealed that strong VEGF signals were detected in the thymic epithelial cells displaying immunoreactivity for CK during thymic regeneration (Fig. 3: a to c). Consistent with previous data [28], the enhanced levels of TrkA protein were observed in cytokeratin (CK)-positive thymic epithelial cells of the regenerating thymus (data not shown). Since both VEGF and TrkA proteins were observed in thymic epithelial cells, we evaluated whether VEGF and TrkA proteins were expressed in the same epithelial cells. The VEGF and TrkA proteins were co-localized in CK-positive thymic epithelial cells (Fig. 3: d to f), although there were some cells positive for TrkA and negative for VEGF and vice versa.

3.4. Up-regulation of VEGF and vWF expression by NGF in vivo

Since NGF/TrkA signaling affects VEGF gene expression in endothelial cells [21,23], we investigated whether the enhanced NGF/TrkA signaling in regenerating thymus can up-regulate the VEGF expression in thymus. Intraperitoneal injection of NGF after CY treatment significantly elevated the VEGF protein levels in the thymus (Fig. 4c, f, and i), compared with PBS-injected (Fig. 4b, e, and h) or normal control (Fig. 4a, d, and g) thymus. We next examined the effects of NGF-mediated VEGF upregulation on stimulating reparative angiogenesis in thymus. Immunostaining for vWF protein, one of vascular markers, revealed that the thymic blood vessel density was highly elevated in NGF-injected thymus (Fig. 4l), compared with PBS-injected (Fig. 4k) or normal control (Fig. 4j) thymus.

3.5. Up-regulation of VEGF expression by NGF in vitro

Since cells immunolabeled for both TrkA receptor and VEGF were identifiable as thymic epithelial cells (Fig. 3), we used thymic
subcapsular/cortex epithelial cell line, SNEC, to confirm the in vivo stimulating effects of NGF on VEGF gene expression in vitro. TrkA transcripts were detected in SNEC (data not shown), suggesting that NGF can trigger intracellular signals to activate the expression of target genes in SNEC. As shown in Fig. 5A, NGF increased VEGF mRNA levels in SNEC by ∼2.5-fold. The levels of VEGF protein were also elevated under the treatment of NGF by ∼4.0-fold (Fig. 5B). The findings from Western immunoblot analysis were further corroborated by immunocytochemistry. As shown in Fig. 5C, the VEGF signal was stronger in NGF-treated cells than in the untreated cells. These results strongly suggested that NGF could function as an upstream signal for activating VEGF gene expression in thymic epithelial cells.

To demonstrate that NGF-mediated VEGF gene induction is a specific receptor-mediated event, we used a selective antagonist of TrkA, K252a. As shown in Fig. 5D, K252a at a nontoxic and selective concentration of 100 nM prevented NGF induction of VEGF gene expression, indicating that the effect of NGF on VEGF gene induction is specifically TrkA-mediated.

4. Discussion

The present results showed that reparative angiogenesis occurs during adult thymic regeneration. Thymic blood vessel density examined by both angiography (Fig. 1B) and PECAM staining (Fig. 1C) was significantly elevated at 3 days after CY treatment, when thymic regeneration begins to occur (Fig. 1A). Therefore, the thymus might be a good model system for studying the molecular basis of reparative angiogenesis that is closely associated with adult tissue regeneration.

Angiogenesis is essential for normal embryonic development and also contributes many pathological states such as tumor growth [1–3, 6]. Although recent studies regarding important roles of angiogenesis in stimulating adult tissue regeneration have been published [4, 5], they do not much address the molecular basis of the reparative angiogenesis during adult tissue repair/regeneration. Recent studies reported that therapeutic angiogenesis stimulated by angiogenic factors is important for ischemic tissue regeneration [34]. Therefore, considering that enhanced reparative angiogenesis accompanies thymic regeneration (Fig. 1), it is likely that the levels of angiogenic factor(s) may be elevated in regenerating thymus. Many angiogenic factors have been known to control blood vessel formation [1–3, 6]. Among them, VEGF is a crucial mediator of vascular hyperpermeability, angiogenesis, and inflammation, processes intimately involved in tissue repair/regeneration [4, 5, 7, 8, 31, 34, 35]. In this study, the dramatic increase of VEGF protein (Fig. 2A) and mRNA (Fig. 2B) was found in the regenerating thymus at 3 days after CY treatment, which is concurrent with the higher density of microvessels (Fig. 1B and C). The strong correlation between up-regulated expression of VEGF and microvessel density suggests that reparative angiogenesis in regenerating thymus might be, at least in part, dependent on VEGF induction.

Neurotrophins (NTs) are a family of secreted growth factors consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [36]. In the thymus, mRNAs for all neurotrophins and their receptors have been detected. The recent studies strongly indicate that NTs control the biology of...
Thymic stromal cells and T cells, including differentiation and survival, and are involved in the thymic organogenesis [37].

It has been shown that the thymic TrkA is necessary in normal thymogenesis [38] and NGF administration produces epithelial cell hypertrophy in vivo [39] and increases the transcription of IL-6, an important mediator of T cell development within the thymus, in epithelial cells in vitro [40].

We have recently demonstrated the up-regulated expression of NGF [27] and TrkA [26] in predominantly thymic epithelial cells during thymic regeneration from acute involution induced by cyclophosphamide in the rat. The presence of NGF but also its upregulated expression was mainly observed in the subcapsular, paraseptal, and perivascular epithelial cells, and medullary epithelial cells including Hassall’s corpuscles in both the normal and regenerating thymus [27]. In the case of TrkA, its expression was found in the subcapsular, paraseptal, perivascular, and medullary epithelial cells in the normal rat thymus. Cortical epithelial cells show very faint or almost no staining. Significantly increased expression of TrkA protein is observed in thymic epithelial cells, primarily in subcapsular, paraseptal, perivascular, and cortical epithelial cells during thymus regeneration following cyclophosphamide-induced acute involution [26]. Also, during corticoid-induced thymic degeneration the cortical epithelial cells that normally lack TrkA express this receptor [41]. These findings indicate that the TrkA receptors on the thymic epithelial cells in the cortex may be more involved in the regenerative mechanisms of the rat thymus resulting from acute involution than the TrkA receptors on the medullary epithelial cells. However, the contribution made by the NGF–TrkA interaction to thymic regeneration remains to be defined.

Since NGF plays a role as an angiogenic factor [16,17,19–23,42,43], it is conceivable that enhanced NGF/TrkA signaling may be responsible for stimulating reparative angiogenesis during thymic regeneration. Interestingly, VEGF protein was co-localized in the TrkA-positive thymic epithelial cells (Fig. 3: d to f), indicating a link between NGF/TrkA signaling and VEGF
synthesis in the thymic epithelial cells. It has been reported that in addition to direct proliferating effects on endothelial cells, NGF exhibits its angiogenic activity by modulating VEGF gene expression via TrkA receptor [17,19,23]. To investigate whether NGF/TrkA signaling system directly controls VEGF gene expression in thymic epithelial cells, we used thymic epithelial cell line, SNEC (subcapsular nurse epithelial cell). It was verified in vitro that NGF directly stimulates SNEC to increase the levels of VEGF mRNA (Fig. 5A) and protein (Fig. 5B and C). Furthermore, in vivo injection of NGF also caused an increase of VEGF protein (Fig. 4c, f, and i) and elevated thymic blood vessel (Fig. 4l) in regenerating thymus. These results indicate that NGF promotes the production of thymic VEGF in vitro and in vivo.

K252a, a well-known antagonist of TrkA [20], reduced the level of NGF-induced VEGF mRNA in SNEC (Fig. 5D), suggesting that NGF control of VEGF induction is via TrkA receptor-mediated signaling pathways. In addition, considering that cells immunolabeled for the TrkA and VEGF were identifiable as thymic epithelial cells, the effect of NGF is probably...
attributable, at least in part, to autocrine release of VEGF in thymic epithelial cells.

TECs, which provide MHC, antigen peptides, and growth factors, play an essential role for intrathymic T-cell development in thymus [28,44,45]. In addition, recent report demonstrated that the ablation of VEGF gene in TECs alone was sufficient to disrupt the network of thymic blood vessels [46], suggesting the important role of TECs for providing instructive cues for the correct spatial organization of the thymic vasculature. Thus, it is likely that NGF-induced VEGF up-regulation in TECs may produce concomitant angiogenesis in the regenerating thymus.

In this study, we showed for the first time that reparative angiogenesis occurs dramatically during adult thymic regeneration that accompanies the up-regulation of VEGF gene expression. We also demonstrated that NGF induces thymic epithelial expression of VEGF both in vitro and in vivo. In summary, our results indicate that the reparative angiogenesis in the regenerating thymus is probably attributable, at least in part, to the NGF-mediated VEGF induction in thymic epithelial cells, suggesting that modulators of VEGF and/or NGF signaling might find usage as therapeutic regulators for the repair of acute, accidental, or age involution of adult thymus by stimulating reparative angiogenesis.

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