The Sialomucin CD34 Is Expressed on Hematopoietic Cells and Blood Vessels During Murine Development

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The processes of angiogenesis and hematopoiesis require a high degree of coordination during embryogenesis. Whereas much is understood about the development of the vascular system in avian embryos, little information has been attained in mammals, predominantly because there are no specific markers for either blood vessels or hematopoietic cells in any developing mammalian system. We have recently shown that murine CD34 (mCD34) is expressed on the vascular endothelium in all organs and tissues of the adult mouse as well as on a small percentage of presumably hematopoietic stem cells in the bone marrow and fetal liver. Here we show that mCD34 is also expressed on the endothelium of blood vessels and on a subset of hematopoietic-like cells throughout murine development. mCD34 is first observed on the yolk sac endothelium of day 7.5 embryos and on a subset of hematopoietic cells within these yolk sacs. mCD34 expression is maintained on vessels and hematopoietic cells in all organs and tissues throughout embryogenesis. In addition, mCD34 is localized on growth cone-like filopodial processes that appear at the budding edge of newly sprouted capillaries. Double staining of capillaries for mCD34 and laminin shows that these growth cone-like processes seem to be free of laminin, whereas the formed capillaries seem to be coated with this extracellular matrix protein. Analysis of vessels in developing brain shows that these filopodial processes seem to be directed toward the ventricular epithelium, a previously described site of vascular endothelial growth factor synthesis. Finally, we show that the vascular structures of developing murine embryoid bodies also express mCD34. These data suggest that mCD34 is a useful marker for the analysis of the development of the blood vascular system in murine embryos.

The development of the blood and blood vascular systems during embryogenesis is a complex process that requires the coordinated spatial and temporal expression of a diversity of cell types. Blood vessel development occurs in situ by two disparate mechanisms termed vasculogenesis and angiogenesis. Vasculogenesis is the de novo production of blood vessels from mesodermal precursors and occurs, for example, in the extraembryonic yolk sac, whereas angiogenesis is the sprouting of new vessels from pre-existing ones that occurs within the embryo proper. In contrast to the in situ development of blood vessels, the initial development of the hematopoietic system as blood islands in the yolk sac is followed by the migration of the hematopoietic site to the para-aortic region, the fetal liver, and ultimately to the bone marrow. It is possible that a single progenitor cell, termed the hemangioblast, gives rise to the vascular and hematopoietic systems early in development, although this hypothesis is based on the observation that both vascular and hematopoietic structures positive for the same monoclonal antibodies (QH-1 or MB-1) seem to arise simultaneously in the yolk sac of avian embryos. Because blood vessel growth often occurs along defined pathways during embryogenesis, it is likely that morphogenetic gradients, perhaps formed by growth or differentiation factors such as the vascular endothelial growth factor (VEGF), are used during development to direct the angiogenic process in the appropriate spatial manner. However, the exact molecular mechanisms that underly the directed vascular growth are presently unknown. Finally, at least some of the molecular mechanisms used during the development of blood vessels in the embryo, particularly the angiogenic budding process, seem to be recapitulated by vessels that invade and nourish tumors.

Whereas many of the current concepts of embryonic hematopoiesis and blood vessel development have been derived from studies of avian embryos, knowledge of such processes in mammalian embryos, and particularly in murine embryos, would have many advantages. For example, the ability to analyze blood vessel development in mice mutated at defined loci, such as those encoding VEGF, or its putative receptors Flt-1 or Flk-1, would provide a great deal of insight into the angiogenic process. Unfortunately, the analysis of blood vessel development in mammalian embryos has been hampered by the lack of specific markers for embryonic blood vessels. Previously, we showed that antibodies against the vascular sialomucin, CD34, recognized all blood vessels in adult mouse tissues as well as a subset of potential hematopoietic progenitors in the bone marrow. Here we provide evidence that CD34 is also expressed on blood vessels during the vasculogenic and angiogenic processes that occur during murine embryogenesis. In addition, we show that CD34 is also expressed on hematopoietic-like cells that may be representative of embryonic blood stem cell progenitors. Finally, we show that CD34 is found on filopodial processes at the growing tip of angiogenic vessels that bear a remarkable morphologic resemblance to neuronal growth cones.

MATERIALS AND METHODS

Antibodies. The anti-mCD34 polyclonal antibody was prepared as previously described. Briefly, recombinant murine CD34 extracellular domain was purified from a murine CD34/IgG chimera after papain cleavage and protein A Sepharose (Pharmacia, Piscataway, NJ) chromatography. The purified material was used to immunize rabbits, and the resultant anti-mCD34 polyclonal antibodies were affinity purified using immobilized recombinant mCD34/IgG. Rat anti-mouse laminin monoclonal antibody was purchased from Gibco.
Fig 1. Anti-CD34 labels vessels in the developing embryo. Embryos of various stages were fixed and labeled with affinity-purified anti-CD34 antibodies. (A) shows the cephalic capillary plexus of a whole-mount 9-day embryo; (B) is a dorsal view of a whole-mount embryo, displaying the intersomitic vessels and adjacent capillary plexus; (C) is a higher magnification view of such intersomitic vessels; (D) is a view of the vessels surrounding the developing bone in the hindlimb of a 13.5 day embryo; (E) shows the vessels surrounding and within the neural tube of a 13.5 day embryo; and (F) shows the staining of vessels developing within the headfolds of an 8 to 8.5 day whole-mount embryo. Scale bars for (A), (D), and (E) are 50 μm; (B), 20 μm; and (C) and (F), 5 μm.

(Grand Island, NY) and fluorescein-conjugated donkey anti-rabbit IgG and Texas Red–conjugated goat anti-rat IgG were purchased from Jackson Laboratories (West Grove, PA).

Embryo dissection, immunofluorescence, and confocal microscopy. Embryos of appropriate stages were dissected from maternal tissue and fixed in 3.7% paraformaldehyde at 4°C overnight. For whole mount preparations, embryos were then rinsed in phosphate-buffered saline (PBS), and blocked for at least 1 hour at room temperature in blocking solution (5% serum; 0.1% Triton X-100 in PBS). Embryos were incubated in primary antisera for at least 1 hour at room temperature (or overnight at 4°C), washed three times in PBS, and incubated in secondary antisera at room temperature for at least 1 hour. Embryos were then washed 3 times in PBS and mounted in 50% glycerol in PBS. The working concentrations of primary antisera were as follows: affinity-purified rabbit anti-mCD34 antibody, 10 to 15 μg/mL; and rat anti-mouse laminin monoclonal antibody, 1:100 dilution. The dilutions of secondary reagents were as follows: fluorescein-conjugated donkey anti-rabbit IgG, 1:200;
and Texas Red–conjugated goat anti-rat IgG, 1:200. For F-actin labeling, embryos were incubated with Bodipy 581/591 phalloidin (Molecular Probes, Eugene, OR) at a working concentration of 1 U/200 μL PBS for at least 20 minutes at room temperature. For cryostat sections, fixed embryos were equilibrated overnight in 0.5 mol/L sucrose and mounted in OCT (Miles Scientific, Naperville, IL). Frozen sections (8 to 15 μm) were collected on Plus slides (Baxter, McGraw Park, IL) and allowed to air dry for at least 1 to 2 hours. Slides were then stored at −80°C until ready for use. The immunofluorescence staining procedure for cryostat sections is as described above for whole mount embryos. Confocal microscopy was performed on a Molecular Dynamics Multiprobe 2001 system (Santa Clara, CA).

Growth, differentiation, and analysis of embryoid bodies. The D3 line of embryonic stem (ES) cells was induced to form embryoid bodies by the removal of leukemia inhibitory factor (LIF) from the

Fig 2. Anti-CD34 labels the vasculature and “progenitor” cells in the embryonic yolk sac. Yolk sacs of embryos of different stages were fixed and labeled with affinity-purified anti-CD34 antibodies (green staining) and, in some cases, Bodipy phalloidin (orange staining) to show the architecture of surrounding cells that are negative for CD34 staining. (A) shows scattered CD34-positive cells in the developing vasculature of the yolk sac of a 7.5-day whole-mount embryo; (B), (C), (D), and (F) are dual-fluorescence images of yolk sacs from 9 to 9.5 day embryos, double-labeled for CD34 (green) and F-actin (orange); CD34 labels both the endothelium as well as hematopoietic cells that exist either as single cells (especially C) or as clusters of cells (B, D, and F). Note the presence of other cells in the lumen of the embryonic yolk sac vasculature (presumably a majority of which represent embryonic erythroblasts) that are negative for CD34 staining but are visible with phalloidin staining. In most cases, the progenitor cells are in close contact with the surrounding endothelium. (E) presents this contact between a pair of mCD34 positive hematopoietic cells and the underlying endothelium at higher magnification. Scale bars in (A) through (D) and (F) are 5 μm; scale bar in (E) is 2 μm.
media and culture on bacteriologic plates. Embryoid bodies were maintained in Dulbecco’s modified Eagle’s medium (high glucose) for up to 17 days at 37°C. Embryoid bodies were fixed and prepared for immunofluorescence essentially as described above for embryos.

RESULTS

Expression of mCD34 in developing embryos. We have previously shown that an affinity-purified polyclonal antibody directed against recombinant murine CD34 (mCD34) specifically recognized blood vessels, including capillaries and postcapillary venules, as well as a small subset of hematopoietic blastlike cells and megakaryoblastic progenitors in the bone marrow of adult mice. As can be seen in Fig 1, this polyclonal antibody also specifically recognizes blood vessels in developing murine embryos. Many of the known blood vessels are stained by this antibody, including, for example, the dorsal aorta and the intersomitic vessels. In addition, many smaller, previously uncharacterized vascular structures are also recognized by the antibody. For example, examination of day 9.5 embryos shows a remarkable capillary plexus distributed throughout the embryo (Fig 1A). Some of the earliest recognizable vessels in the embryo, such as those in the head fold of a 8 to 8.5-day embryo, are positive for CD34 staining (Fig 1F). The antibody also stains

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Fig 3. Identification of CD34+ presumptive progenitors within the embryo. Sections of 13 to 13.5-day embryos labeled with anti-mCD34 antibody. (A) and (B) represent sections through the developing fetal liver. Note the somewhat flattened morphology of CD34-positive nonendothelial cells within the tissue of the liver as opposed to CD34-positive cells outside of the tissue but within vessels of the liver. An arrowhead shows one of the nonendothelial CD34-positive cells. (C) represents cells within the paraaortic region of the embryo. (D) shows cells located in the region of the developing bone marrow. In both (C) and (D), arrowheads show nonendothelial CD34-positive cells. Scale bar for all panels is 5 μm.
the endothelium of large vascular structures, including the aorta and the heart (data not shown). The earliest recognizable embryonic structures that are stained by anti-mCD34 are the mesodermal cells of the developing yolk sac. As can be seen in Fig 2A, these structures are mCD34 positive at very early times of embryonic development (~day 7.5) and may correspond to the presumptive hemangioblastic progenitor cell type. These results show that mCD34 is expressed on very early blood vessels of the embryo. Thus, it seems that anti-mCD34 is an effective marker for blood vessels during murine embryogenesis.

Expression of mCD34 on presumptive hematopoietic progenitor cells of the embryo. Examination of yolk sacs of day 8.5 to 9.5 embryos showed that both endothelial cells as well as nonendothelial, hematopoietic-like cells within the yolk sac were mCD34 positive (Fig 2). Previous isolation
of CD34-positive cells from human and primate bone marrow as well as from murine bone marrow and fetal liver has shown that these cells contain a CD34-positive stem cell population that can give rise to all hematopoietic lineages in lethally irradiated recipients. It therefore seems likely that the mCD34-positive cells that are found within the yolk sac are the presumptive hematopoietic progenitors of the embryo, and we have recently shown that the nonadherent, CD34-positive fraction purified from day 9 to 10 yolk sacs contains progenitor activity as measured in methyl cellulose culture assays. Analysis of random sections of yolk sacs from day 9 embryos showed that there were approximately 3,000 mCD34-positive hematopoietic cells per yolk sac (data not shown). mCD34-positive yolk sac hematopoietic cells are often seen to be clustered together and in physical contact with the mCD34-positive endothelial cells of the yolk sac (Fig 2). Hematopoietic cells are also seen in other regions later in embryonic development. For example, mCD34-positive nonendothelial cells are seen in the fetal liver of day 14 embryos (Fig 3), a known site of hematopoiesis in a diversity of embryos. Interestingly, these nonendothelial, potentially hematopoietic cells in the fetal liver seem to have a different, less rounded, and more “adherent” morphology than the yolk sac hematopoietic cells. In addition, we also have observed presumptive hematopoietic progenitor cells in the bone marrow and para-aortic regions of embryos as well (Fig 3). These presumptive hematopoietic progenitors in fetal liver, bone marrow, and the para-aortic region seem to be on the outside of the mCD34-positive blood vessels, in contrast to the yolk sac where the hematopoietic cells were lumenedly disposed, consistent with the possibility that these putative hematopoietic progenitor cells intravasate into the fetal circulation during maturation. In summary, the mCD34 antigen seems to be on a small subset of presumptive hematopoietic progenitor cells at all of the known hematopoietic sites during the development of the embryo.

Expression of mCD34 on filopodial processes of budding capillaries. Previous data on developing rat brain capillaries and on avian vessels differentiating in vitro showed filopodial structures at the growing end of angiogenic (sprouting) vessels. Examination of vessels in murine embryos from a number of developmental stages shows that these filopodial processes are mCD34 positive and occur at a diversity of sites throughout the embryo. Figure 4 shows several examples of such structures that are predominately found at the growing tips of capillaries during the angiogenic process. As previously proposed, the processes are highly reminiscent of the growth cone structures that are seen at the guiding end of neurons as they progress through the extracellular matrix, presumably in response to guidance cues. Double staining of sprouting vessels with antibodies against mCD34 and the extracellular matrix protein, laminin, shows that the filopodial structures are free of this protein, whereas the formed vessels are surrounded by it (Fig 5). Because adhesive interactions between extracellular laminin and endothelial integrins might be expected to solidify the formation of vascular pathways, these results also suggest a more dynamic role for the growth cone-like structures and filopodial extensions. The possibility that filopodial processes are involved with the guidance of blood vessels is also suggested by an analysis of vessels in the developing brain. Figure 6 shows that filopodial processes emanate from capillaries at the pial surface and extend toward the ventricular epithelium. Previous in situ hybridization data have shown that mRNA for VEGF forms a gradient from the ventricular surface outward toward the pial surface. This observation is consistent with the possibility that the filopodial processes of brain capillaries are directed toward a gradient of VEGF. In summary, sprouting vessels in the developing murine embryo seem to contain growth cone-like structures decorated with long filopodia that may be involved with the formation of appropriate vascular pathways during development.

Expression of mCD34 on vascular structures in embryoid bodies. Previous studies have suggested that vessels and hematopoietic cells, both reminiscent of yolk sac structures, develop when ES cells are induced to differentiate after the removal of LIF. Whereas the vascular structures observed in these developing embryoid bodies seemed to be blood vessels, the lack of a marker for these structures made a definitive identification difficult. As can be seen in Fig 7, staining of embryoid bodies shows vascular structures that are clearly mCD34 positive. The mCD34-positive vessels in the embryoid bodies seem to be poorly formed and less organized than, for example, vascular structures that are seen in normally developing embryos. In stark contrast to the well-developed growth cone-like structures of the vessels during embryonic development, vascular structures in the embryoid bodies do not show such morphologically distinct entities (Fig 7). These data suggest the possibility that the lack of organization in the vascular system of the embryoid body may, at least in part, be caused by a lack of proper guidance cues that may interact with or induce the growth cone-like structures that are normally found in the sprouting vessels of developing embryos.

DISCUSSION

The complex interplay between early vascular and hematopoietic progenitor cells, the extracellular matrix, and various growth and guidance/differentiation factors that ultimately gives rise to the blood and circulatory systems of the fetus is a remarkable developmental processes. Studies in the avian embryo have shown that the development of the vascular system arises by a combination of vasculogenic processes, in which vessels develop de novo from undifferentiated mesodermal progenitors, and angiogenesis, in which vessels bud from pre-existing capillaries. Data from both the avia and murine systems have shown that embryonic hematopoiesis initially occurs in the yolk sac and later migrates intra-embryonically to the para-aortic region, the fetal liver, the spleen, and the bone marrow. A great deal of morphologic information has been gained from the work on avian embryos, the molecular mechanisms involved in the development of the blood and vascular systems during embryogenesis are not well understood. This is in part because of the fact that molecular probes for the various avian genes that might be involved with these processes have not yet been obtained. In addition, the ability to
manipulate the avian genome in a way that has been recently accomplished in the mouse has yet to be achieved. The development of reagents, such as the anti-mCD34 antibodies described here, that will allow for an analysis of the development of the blood and blood vascular systems in murine embryos may therefore be of great potential use. Much of the work reported here on murine vascular and hematopoietic development is similar to that previously reported in the avian system. For example, we have observed both vasculogenic (yolk sac) and angiogenic (capillary budding) processes of blood vessel development throughout murine embryogenesis. As was previously found in avian yolk sacs, both endothelial cells and hematopoietic-like cells carry a common marker. Whereas in the case of avian cells the molecular nature of the common marker is undefined, the marker in murine embryos is the sialomucin CD34. The finding of mCD34 on both vascular and hematopoietic cells is consistent with the hypothesis that a single mesodermally derived cell type, the hemangioblast, differentiates to give rise to vascular endothelium as well as to hematopoietic progenitor cells of the yolk sac. The observations reported here in mice, and previously in birds, that vessels and a subset of hematopoietic cells within the more advanced embryo (ie, not yolk sac–derived vascular or hematopoietic cells) are also positive for either mCD34 or the unknown avian surface marker recognized by the monoclonal antibodies MB-1 or QH-1 is also suggestive of the possibility that both of these intra-embryonic cell types may be derived from a single progenitor hemangioblastic stem cell. Isolation of such a bipotential progenitor cell from human bone marrow has been reported, and is currently controversial. It will be of interest to determine if this type of progenitor can be isolated from murine embryos.

The observation that a subset of murine yolk sac, fetal liver, para-aortic region, and fetal bone marrow cells are positive for mCD34, together with the previous demonstrations that CD34-positive cells from the bone marrow of adult humans, primates, and mice are pluripotent hematopoietic stem cells, suggests that the embryonic mCD34 positive hematopoietic cells are also blood stem cells. Previous investigators have shown that a small subset of hematopoietic cells in murine yolk sacs and fetal liver can give rise to a diversity of hematopoietic progenitors. Whereas we have not yet formally proved that the mCD34-positive embryonic cells are pluripotent hematopoietic progenitors, by analogy with the adult human, primate, and murine bone marrow, this seems likely. In addition, we have recently shown that the nonadherent, mCD34-positive cells purified from yolk sacs can give rise to a number of different hematopoietic lineages in vitro in response to a diversity of growth factors.

The various morphologic findings reported here may therefore be of some importance for hematopoiesis. Thus, the clustering of mCD34-positive cells and their apparent adhesive interaction with the mCD34-positive endothelium of the yolk sac suggests that these interactions may have functional significance for the development of yolk sac hematopoietic cells. If true, this would suggest that the yolk sac endothelium might be a useful source for various growth and differentiation factors that are required for hematopoietic stem cell proliferation and differentiation. An additional interesting observation regards the morphology of the mCD34-positive cells in the fetal liver. In contrast to the rounded appearance of the mCD34-positive yolk sac cells, the fetal liver cells seem to be flattened and apparently more "adherent." The significance of this flattened morphology is presently unknown, but it also suggests the involvement of adhesive interactions between the hematopoietic progenitors and the stromal cells of the fetal liver that are required for stem cell proliferation and/or differentiation. It will be interesting to determine if adhesion molecules that function in adult bone marrow, such as very late antigen–4 and vascular cell adhesion molecule–1, also function during embryogenesis.

Vessels often follow highly specific pathways during embryonic differentiation. For example, in avian vascular development intersomatic arteries bud off the dorsal aorta, grow between the somites, and then make an abrupt turn to the right and grow until they fuse with the adjacent intersomatic capillary. This type of specific guidance is reminiscent of neuronal guidance, especially in insect embryos, in which it has been studied to the greatest extent. Whereas many of the molecular cues involved with insect neuronal guidance are understood, little is currently known about vascular pathway formation. Thus, it is interesting that the same type of morphologic entity, the growth cone, is found on both the growing tip of the axon and the angiogenic budding capillary. The observations reported here suggest a number of interesting aspects regarding this growth cone-like entity. First, the vascular growth cone is always found on the growing end of the budding capillary. This result is consistent with the hypothesis, originally proposed for neuronal growth cones, that this structure may be involved with the sensing of guidance cues during vessel growth and pathfinding. The highly extended filopodial structures that we have observed here would provide for a mechanism to sense both immobilized guidance cues as well as the proximity of other vascular structures. We also found that the extracellular matrix protein, lammin, surrounded the formed vessels but was not in proximity to the growth cones. Adhesive interactions between endothelial integrins and extracellular laminin could cement vascular pathways, and the lack of laminin on vascular growth cones suggests that these structures might have a greater degree of mobility, although it is also possible...
Fig 7. Anti-CD34 labels capillary-like structures in embryoid bodies. Cystic embryoid bodies developing in vitro were fixed and labeled with anti-mCD34 antibodies. (A) is a low magnification view of a field of embryoid bodies and the associated CD34 labeling. (B) and (C) are higher magnification views of vessels within embryoid bodies. In general, vessels appear disorganized, and although there are some filopodial processes present, they lack the elaborate organization displayed by embryonic capillaries. Scale bar in (A) is 20 μm; scale bar in (B) is 10 μm; scale bar in (C) is 5 μm.
that other extracellular matrix proteins such as collagen or fibronectin interact with integrins on the growth cone structures.

Analysis of mCD34 expression in the developing brain may give some indication of the types of guidance cues that vessels use for the formation of specific pathways. Examination of brains of day 13 embryos showed that the filopodial projections and, in some cases, capillaries, that were derived from vessels on the pial surface were predominantly directed toward the ventricular surface of the brain. These data were consistent with the possibility that some sort of immobilized guidance cue was deposited on the ventricular surface that attracted the directed growth of the filopodial structures and capillaries.\(^{15}\) In situ hybridization analyses by Breier et al have shown that VEGF mRNA is produced by cells of the ventricular endothelium.\(^{12}\) Thus, it is possible that a gradient of immobilized VEGF is detected by receptors on the filopodial projections that emanate from capillaries in the pial layer of the brain. Contact between receptors on the extended filopodia and immobilized VEGF might then activate a guidance program in the vessel with the result that capillaries would than bud toward the VEGF gradient. If true, it would follow that mice that are deficient in the production of VEGF should therefore have deficiencies in the directed growth of blood vessels, particularly in the brain.

Finally, some comments should be made regarding the possible function of mCD34 on hematopoietic progenitors and vessels during murine development. In adult peripheral and mesenteric lymph nodes, endothelial mCD34 seems to function as a ligand for the L-selectin cell adhesion molecule on the lymphocyte cell surface.\(^{34}\) In this case, the mucinlike mCD34 seems to present sulfated, sialylated carbohydrates to the lectin domain of L-selectin, thus mediating cell adhesion between lymphocytes and the endothelium of peripheral lymph nodes.\(^{35\text{-}36}\) The broad vascular expression of mCD34 in other vascular sites of the adult is consistent with a role for this mucin in L-selectin-mediated adhesion in nonlymph node sites, although this possibility has not yet been investigated.\(^{16}\) The function of mCD34 on hematopoietic progenitors has not yet been determined, but proteolytic removal of the mucin from the cell surface of human hematopoietic progenitor cells does not affect their ability to proliferate in vitro.\(^{37}\) Because very little leukocyte trafficking occurs in the embryo, it is highly unlikely that vascular mCD34 functions as a scaffold for the presentation of carbohydrate ligands to L-selectin. One possible function for vascular mCD34 could be to present carbohydrate ligands to E-selectin during capillary formation, because it has been recently reported that this endothelial selectin is involved with this angiogenic process.\(^{38}\) However, the recent production of a mouse that lacks E-selectin but develops normally (B. Wolitzky, personal communication, January 1994) as well as a rare genetic defect of humans with apparently normal vascular lature in which the sialyl Lewis\(^\text{a}\) carbohydrate ligand for E-selectin is deficient\(^{39,40}\) both seem to argue against a role for E-selectin–mCD34 carbohydrate interactions in capillary development. The cytoplasmic domain of CD34 can be phosphorylated in response to protein kinase C activation, so it is also possible that this molecule is involved with signal transduction.\(^{41}\) Thus, it will be of great interest to examine the hematopoietic and vascular development of embryos and embryoid bodies that have been made null for mCD34 expression.\(^{29}\)

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