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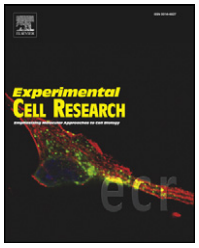

Research Article

Angiopoietins-1 and -2 play opposing roles in endothelial sprouting of embryoid bodies in 3D culture and their receptor Tie-2 associates with the cell–cell adhesion molecule PECAM1

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ABSTRACT

Angiopoietins 1 and 2, ligands for the receptor kinase Tie-2, have been proposed to play critical but opposing roles in vascular development. Since signaling by Tie-2 is likely affected by other endothelial cell receptors such as Flk-1, the receptor for VEGF, and cell–cell adhesion receptors PECAM1 and VE-cad, we explored their interactions in a 3D model of vasculogenesis. When murine embryoid bodies (EBs) were treated with VEGF in Matrigel in the presence or absence of Ang-1 or Ang-2 for eight days, Ang-1 abrogated vascular sprouting for treatments started at days 0 or 3. In contrast, Ang-2 greatly accelerated vascular sprouting compared to untreated EBs. These results were confirmed in a second model system where VEGF treated HUVECs were grown in Matrigel in the presence or absence of Ang-1 or Ang-2. Since vascular sprouting must be precisely controlled in the developing embryo, it is likely that cell–cell adhesion molecules play a role in sensing the density of vascular sprouts. In this respect, we have shown that PECAM1 and CEACAM1 play essential roles in vascular sprouting. We now show that PECAM1 is associated with Tie-2, becomes phosphorylated on its ITIMs, and recruits the inhibitory phosphatases SHP-1 and SHP-2. In addition, PECAM1 is associated with VE-cad and may similarly regulate its signaling via recruitment of SHP-1/2.

Introduction

Vasculogenesis, the generation of the vascular system in the developing embryo, involves coordinated signaling among cell surface receptors that respond to environmental signals. While VEGF and its receptor Flk-1 are among the most studied [1], other ligands such as Angiopoietins-1 and -2 (Ang-1 and Ang-2) and their receptor Tie-2, also play critical roles [2,3]. In addition, the extent of vascular sprouting is controlled by cell–cell adhesion molecules such as PECAM1 [4], CEACAM1 [4] and VE-cad [5,6]. In an effort to determine the possible interactions among these receptors, we developed a 3D model of vasculogenesis in which VEGF treated murine embryonic stem cells differentiated into embryoid bodies (EBs) undergo vascular sprouting when transferred to 3D cultures containing Matrigel, a source of extracellular matrix. The sprouting was inhibited and the vessel architecture was abnormal when EBs were treated with either anti-PECAM1 or anti-CEACAM1 monoclonal antibodies prior to embedding in Matrigel, while identical treatment with an antibody isotype control had no effect [4]. These results suggest that inhibition of cell–cell signaling affected the function of many of the critical receptors for endothelial cells. In an effort to understand these

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interactions, we tested the response of EBs to Ang-1 and Ang-2 and the possible association of Tie-2 with PECAM1 and CEACAM-1.

Tie-2 (endothelial cell-specific tyrosine kinases 2) is a receptor tyrosine kinase highly expressed on endothelial cells with wide-ranging effects that include angiogenesis, inflammation, and vascular extravasation [2,3]. Genetic ablation of Tie-2 is embryonic lethal due to specific defects in the formation of primary capillary plexus and higher order branching vessels [7]. Ang-1 is mainly produced by vascular mural cells, whereas endothelial cells are the main producers of Ang-2 [3]. However, the regulatory roles of Ang-1 and Ang-2 in angiogenesis are controversial with Ang-1 behaving as both an agonist and antagonist [8]. Nonetheless, the absence of Ang-1 causes severe vascular abnormalities in the developing mouse embryo [9], and over-expression of Ang-2 in transgenic mouse embryos leads to a major disruption of the developing vessels [10]. Since Tie-2 was down regulated in our model system, it is a good candidate for negative or positive regulation by Ang-1/2 and negative regulation by either PECAM1 or CEACAM1, both of which possess ITIMs.

PECAM1 is expressed on both immature and mature endothelial cells mediating cell–cell adhesion through either homotypic or heterophilic interactions [11,12]. PECAM1 is a member of the Ig-gene super family, comprising 6 extracellular Ig-like domains with a molecular weight of 110–130 kDa [13]. The cytoplasmic domain contains two ITIM motifs that when phosphorylated by a Src kinase in endothelial cells associate with β-catenin and recruit SHP-2, an inhibitory phosphatase that has been shown to dephosphorylate tyrosine phosphates on β-catenin [14,15]. Due to its association with β-catenin, a key component of adherent junctions [14,16] and its recruitment of SHP-2 [17], PECAM1 may play a role in the inhibition of the formation of adherent junctions in endothelial cells. An inhibitory function may be important in the early stages of vascular sprouting when new sprouts invade the extracellular matrix. Given this possibility, it was important to include β-catenin in the list of targets for PECAM1 regulation. In support of this idea, PECAM1 was up-regulated in our model system [4] and is a well-known effecter of both vasculogenesis and angiogenesis [18].

Like PECAM1, CECAM1 has a variable number of Ig-like extracellular domains (2 or 4 in the case of murine CEACAM1) that participate in homotypic and heterotypic cell–cell adhesion [19] and has a cytoplasmic domain containing two ITIMs. Its ITIMs may be phosphorylated by a Src kinase and recruit SHP-1/2 tyrosine phosphatases [20]. CEACAM1 can also associate with β-catenin [21], in addition to a number of cell surface receptors on leukocytes [22–25]. Although CEACAM1 is expressed on newly developing endothelial cells, it is not expressed on mature endothelial cells [26,27]. Thus, although CEACAM1 and PECAM1 share many features in common, they differ in their temporal expression on endothelial cells.

Vascular endothelial-cadherin (VE-cad) is a third critically important receptor on endothelial cells. Genetic ablation of VE-cad leads to embryonic lethality due to failure of development of a mature vascular system [5,6]. VE-cad is a type I transmembrane protein expressed throughout the vascular endothelium and has been reported to be involved in both vasculogenesis and angiogenesis [28]. Since VE-cad negatively regulates Flk-1 [29] and its expression follows that of Flk-1 in our model system, it is likely that VE-cad is held in check during the early stages of vascular sprouting when proliferation predominates. Thus, VE-cad is another possible candidate for regulation by inhibitory co-receptors such as PECAM1 and/or CEACAM1.

The possible association of PECAM1 and CEACAM1 with β-catenin, Tie-2, and VE-cad, as well as their ITIM phosphorylation and recruitment of SHP1/2 phosphatases was assessed by immunoprecipitation with antibodies to each of these molecules. We demonstrated that both PECAM1 and CEACAM1 were tyrosine phosphorylated during vascular sprouting and that CEACAM1 recruits SHP-1, while PECAM1 recruits both SHP-1 and SHP-2. Furthermore, Tie-2 is associated with PECAM1, but not CEACAM1, suggesting that their functions do not overlap. We demonstrated that VE-cad is associated with PECAM1, but not CEACAM1. The novel interaction of PECAM1 with VE-cad was further confirmed in human HUVEC cells, suggesting that this is a general interaction between PECAM1 and VE-cad. The association of PECAM1 with Tie-2 was assessed by treatment of ES cells with Ang-1 or Ang-2, presumptive antagonist or agonist, respectively, to Tie-2. Ang-1 completely inhibited while Ang-2 greatly accelerated vasculogenesis. Furthermore, Ang-2 promoted while Ang-1 inhibited Tie-2 phosphorylation. Thus, we conclude that the inhibitory role of PECAM1 is essential to control the degree of vasculogenesis at the level of both Tie-2 and VE-Cad, and that Ang-1 and Ang-2 oppose each other via the endothelial cell receptor Tie-2. Therefore, PECAM1 may operate by inhibition of both the Tie-2 and VE-cad signaling pathways in vasculogenesis. The pathway(s) regulated by CEACAM1 in this model system require further study.

Material and methods

Antibodies

Goat IgG1, Mouse IgG1, Rat IgG2a and Rabbit IgG1 purchased from eBioscience (San Diego, CA) used as isotype controls. Antibodies used for IPs and/or western blots were: anti-murine CEACAM1, CC1 (mouse IgG1), a kind of gift of Dr. Holmes (University of Colorado Health Sciences, CO); anti-human CEACAM1, T84.1 (mouse IgG1) developed at City of Hope; anti-mouse PECAM1 (Rat IgG2a), anti-mouse β-catenin, anti-phospho-tyrosine (4G10), anti-mouse Tie-2, and anti-mouse Flk-1 from Millipore (Billerica, MA); anti-human PECAM1, anti-human Flk-1, anti-human VE-cad, anti-mouse VE-cad, and anti-human Tie-2 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse SHP-1 and SHP-2 obtained from BD Bioscience (Rockville, MD).

Embryoid body formation

Mouse embryonic stem cells derived from strain 129S1-SVImj were cultured on amitotically inactivated STO-Neo/LIF feeder layer [from EJ Robertson] in DMEM (Mediatech Inc, Herndon, VA) containing 16% fetal bovine serum, non-essential amino acids (GIBCO, stock solution diluted 1:100, final concentration 100 nM), GlutaMax (Invitrogen), penicillin/streptomycin, and 30 nM 2-mercaptoethanol (ES medium). The ES cells were digested at 37 °C with Trypsin/EDTA, and reduced to a single-cell suspension by repeated pipetting. The STO feeder cells were depleted by incubating the cell suspension in a plastic tissue-culture dish in ES medium in a 5% CO2 incubator at 37 °C for 45 min. The non-adherent cells were transferred to a fresh plastic tissue-culture dish, and incubated in a 5% CO2 incubator at 37 °C for an additional 45 min. The resulting non-adherent cells