Vascular smooth muscle cell expression of ectonucleotidase CD39 (ENTPD1) is required for neointimal formation in mice

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Abstract Vascular smooth muscle cell (VSMC) migration and proliferation are critical steps in the pathogenesis of atherosclerosis, post-angioplasty restenosis, neointimal hyperplasia, and chronic allograft rejection. Extracellular nucleotides are known to influence both migration and proliferation of VSMC. Although it is well established that vascular endothelial Cd39/ENTPD1 regulates blood nucleotide concentrations, whether Cd39 associated with VSMC also impacts vascular wall pathology in mice has not been investigated. The objective of this paper is to determine levels of expression of Cd39 on VSMC and functional consequences of gene deletion in vitro and in vivo. Cd39 is the major ectonucleotidase in VSMC, as shown by substantive decreases in ecto-ATPase and -ADPase activity in Cd39-null cells compared to wild type. Significant decreases in neointimal lesion formation are observed in Cd39-null mice at 21 days post arterial balloon injury. Stimulated Cd39-null VSMC have pronounced proliferative responses in vitro. However, using Transwell systems, we show that Cd39-null VSMC fail to migrate in response to ATP, UTP, and PDGF. Cd39 is the dominant ectonucleotidase expressed by VSMC. Deletion of Cd39 in mice results in decreased neointimal formation after vascular injury and

is associated with impaired VSMC migration responses in vitro.

Keywords Cd39 · ENTPD1 · Neointimal formation

Introduction

Accumulation of vascular smooth muscle cells (VSMC) in the vascular neointima is a pathological hallmark of atherosclerosis, restenosis after angioplasty, and vasculopathic rejection of allografts. Purinergic type-2 (P2) receptors, as expressed by VSMC, are activated in an autocrine or paracrine manner by extracellular nucleotides that are released from perivascular nerves, activated platelets and from injured endothelial cells (EC) [1–5].

In vitro studies of VSMC have shown that extracellular nucleotides induce cell cycle progression and proliferation [6–8]. For example, UTP has chemotactic effects on VSMC migration which involve Rho and mitogen-activated purine signaling via P2Y receptor subtypes [3, 9–11].

Ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39 or ENTPD1) is a key vascular endothelial ectoenzyme which hydrolyses extracellular nucleoside tri- and diphosphates in the blood [12–14]. CD39 is expressed on the luminal surface of EC and VSMC [15] and has major thromboregulatory properties that maintain blood flow and fluidity [16, 17]. CD39 over-expression has been shown to provide protection against graft rejection [18], ischemia–reperfusion injury [19], and is involved in angiogenesis [20].

The precise nature and role of CD39 expression on VSMC has been undetermined to date. In the present

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investigation, we determined levels of Cd39 expression on VSMC, the effects of Cd39 deletion on function (in vitro) and evaluated how altered nucleotide phosphohydrolysis pathways impact repair of injured arterial vessels (in vivo) following balloon injury in mice.

Methods

Reagents

DMEM/F-12, trypsin, FCS, penicillin, and streptomycin were obtained from Invitrogen/Gibco (Carlsbad, California). ATP, ADP, and UTP (Sigma) was dissolved in distilled water adjusted to 10 mM, neutralized to pH 7.4 sterilized by filtration and stored at -20°C . Rat PDGF-BB (Sigma) dissolved in distilled water adjusted to 50 nM concentration.

Animals

Adult male (6–10 week) C57BL6 mice (Jackson Laboratory), and *Cd39*^{-/-} mice (C57, BL6) were used for primary VSMC cultures and arterial wire injury. *Cd39*^{-/-} mice were generated in our laboratory as described before [21]. Animals were housed in accordance with the guidelines from the American Association for Laboratory Animal Care and the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (IACUC) approved all research protocols.

Wire injury model

Animals were anesthetized with intraperitoneal ketamine and xylazine. Through a midline neck incision, the common carotid artery was dissected and exposed; proximal and distal vascular controls were obtained. The left external carotid artery was tied off distally, and via transverse arteriotomy, a 0.014-in. flexible angioplasty guidewire was advanced 1 cm. Uniform endothelial denudation was achieved by three passes along the common carotid artery using a rotating motion [22].

Histomorphometric analysis

Animals were euthanized 21 days after arterial injury, and the carotid arteries and contralateral samples were collected and snap-frozen in liquid nitrogen. Samples were sectioned (5 μm) at 200 μm intervals, and stained with hematoxylin–eosin. Six images per vessel were acquired at various magnification levels. Distances and areas of intimal and medial layer were calculated by digital imaging software (Image J, NIH) using arbitrary parameters. Histomorphometry was analyzed in a blinded fashion by two observers.

Immunohistochemistry

Vessels were quick-frozen and stored at -80°C . Cryostat sections were fixed with acetone. Staining was performed using primary antibodies directed against SMC- α -actin (Sigma-Aldrich) and CD31 (BD Pharmingen), as described [23]. Anti-murine NTPDase1 and NTPDase2 polyclonal antibodies (pAbs) were derived by cDNA immunization, as described [23].

Western blot analysis

Cells were serum-starved and protein extracts from cell lysates, and anti-murine NTPDase1 polyclonal antibodies were utilized for Western blotting, as previously described [23].

Cells

Primary mouse aortic VSMC from control and *Cd39*-null mouse were isolated and cultured as previously described [24]. At least three different cultures were used for each group. Cell types and purity were confirmed by α -actin staining. Experiments were repeated with cells isolated from the wild-type and knock-out animals. All experiments using VSMCs were conducted in cell fourth passages or less under subconfluent conditions.

Ectonucleotidase assays

To determine the 5'-tri and di-phosphate nucleoside activities, the 24-well microplates containing VSMC were washed three times with incubation medium in absence of nucleotides/PO4. The reaction was started by the addition of 200 μl of the incubation medium containing (final concentration) 2 mM CaCl_2 , 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4, and various concentrations of nucleotides (ATP, ADP, and UTP) at 37°C . The nucleotide concentrations were chosen from substrate curves to assure the enzyme saturation and the incubation time was chosen from time courses (30 min) to assure the linearity of the reaction (data not shown). To stop the reaction, an aliquot of the cell incubation medium was withdrawn and transferred to an Eppendorf tube containing trichloroacetic acid (TCA) to a final concentration of 5% (w/v).

The production of inorganic phosphate (Pi) was measured using the malachite green method [25], with KH_2PO_4 as the Pi standard. The non-enzymatic Pi released from nucleotide into the assay medium without cells and Pi released from cells incubated without nucleotide were subtracted from the total Pi released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity was then expressed as nanomole Pi released per minute per milligram of protein.

Fig. 1 **a–b** Immunostaining of mouse carotid artery with anti-murine NTPDase1 and NTPDase2 polyclonal antibodies. **a** NTPDase 1 (Cd39) is predominantly expressed on the endothelial and the intimal surface. **b** In contrast, NTPDase 2 (Cd39L1) is mostly expressed on

the adventitia layer of the vessels. **c** Western blotting of VSMC shows that Cd39 is expressed by the wild-type (WT) VSMC and is clearly absent in Cd39-null (knock-out) VSMC

Proliferation assays

Primary mouse VSMCs from control and *Cd39-null* mice were seeded on 96-well plates (1×10^4 cells/well) and cultured for 24 h in DMEM/F-12 containing 10% FCS. Cells were serum-starved (0.2% FCS) for the following 24 h and then stimulated with 10% FCS containing medium, 50 nM PDGF or various concentrations of ATP for 24 h. In studies that cells were stimulated with ATP only for 5 min; cells were kept in 0.2% FCS for completion of 24 h. Twelve hours prior to harvest, cells were pulsed with [3 H]-thymidine (1 mCi/well; Perkin Elmer, Boston, Massachusetts). Experiments were repeated three times in triplicate.

Migration assay

Transwell system (Costar) was used to perform migration assay. Briefly, cells were trypsinized, washed, and resuspended in serum-free medium DMEM containing nucleotides and growth factors at various concentrations. Lower surfaces of chambers were coated with collagen type IV and 250,000 cells were allowed to adhere and migrate over a 6-h period. After this time, cells were fixed with ethanol and stained with 0.2% crystal violet solution. Cells were wiped away from the top of Transwell filters before counting cells on the bottom (i.e., those cells with nuclei that had migrated across the filter). Five fields of vision ($\times 20$ objective) per well were randomly chosen and cell counts performed manually. Data were also normalized to the control wild-type VSMC migration levels.

Statistics

Student's *t*-test with one-tailed distribution was used to analyze data.

Fig. 2 Biochemical activity of intact murine VSMC (expressed as nanomole per minute per milligram). NTPDase activities of intact cells were determined by measurement of phosphate release from the respective substrates. Data are shown as for specific ATPase and ADPase activities in wild type (WT) and *Cd39-null* VSMC (mean $\pm$ SD). *Cd39* is the dominant ectonucleotidase in VSMC and its deletion in null cells significantly decreases ATPase and ADPase activity (* $P < 0.05$)

Results

Murine carotid artery expression of ectonucleotidase

The patterns of *Cd39* or ENTPD1 expression in murine arteries are comparable to those observed in human arterial samples. Specifically, *Cd39* is predominantly expressed on the endoluminal surface of the carotid artery and in the vascular smooth muscle of the media (Fig. 1a). In contrast, ENTPD2 (*Cd39L1*) is expressed on the adventitia layer of the vessels (Fig. 1b).

Cd39 is the dominant VSMC ectonucleotidase

Specific expression of *Cd39* in wild-type VSMC and the complete absence of this ectonucleotidase in *Cd39-null* VSMC were confirmed by Western blot (Fig. 1c). We then

determined relative functional contributions of *Cd39* to the total NTPDase activities in VSMC from wild-type and knock-out cells. Both ATPase- and ADPase-specific activities were significantly decreased in *Cd39-null* VSMC: ATPase specific activity; WT, 28 ± 3 vs. KO, 8 ± 4 nmol/mg/min, $P < 0.002$; ADPase-specific activity; WT, 18 ± 3 vs. KO, 2 ± 1 nmol/mg/min (Fig. 2), $P < 0.003$. This proportionate decrease in biochemical activity in VSMC is comparable to that seen in *Cd39-null* EC [21]. This near absence of NTPDase activity in *Cd39-null* VSMC indicates that *Cd39* is the dominant ectonucleotidase.

Cd39-null carotid arteries have a blunted neointimal response to traumatic injury

A carotid artery wire injury model was used to observe *Cd39* function in post-traumatic vascular injury conditions in vivo.

Fig. 3 Histopathologic features of injured common carotid artery in wild type and *Cd39-null* mice. **a–c** H&E staining of control: WT-uninjured (KO-uninjured had similar features and is not shown here), WT- and *Cd39* KO-injured carotid arteries (CA) 3 week after injury:

KO vessels show less neointimal formation (*I* intima, *M* media). **d–e** Immunostaining of injured vessels with a smooth muscle actin monoclonal Ab. (**d**) and with CD31 monoclonal Ab to the endothelial marker (**e**) confirm that neointimal layer contains VSMC cells

Injured and uninjured-control contralateral carotid arteries of wild-type (WT; $n=6$) and *Cd39*-null ($n=6$) animals were harvested 21 days after injury to evaluate the morphology and neointimal response (Figs. 3 and 4). Control uninjured vessels from both group showed similar features and a sample vessel from the WT group is presented in the Fig. 3a. Although there was slight increase in the WT media (M) surface area this difference was not statistically significant ($P=0.09$). In contrast, KO intimal (I) arterial surface area was significantly less than WT ($P<0.05$) (Fig. 4a). These findings suggest increased proliferation of the *Cd39*-null VSMC within the vessel media but lack of migration to the intima.

Significant decreases in the neointimal lesion formation were observed in *Cd39*-null mice at day 21 after injury (I/M, 0.78 ± 0.3 in WT vs. 0.3 ± 0.1 in the KO, $P<0.05$; Fig. 4b). These neointimal lesions contain alpha-smooth muscle actin positive cells (Fig. 3d).

Fig. 4 Quantitative histomorphometry measurement post-wire injury of common carotid artery in wild type and *Cd39*-null mice. Twenty-one days after arterial injury, carotid arteries were collected from six animals of each group (WT and KO) and snap-frozen in liquid nitrogen. Samples were sectioned (5 μ m) at 200 μ m intervals, and stained with hematoxylin–eosin. Six images per vessel were acquired at various magnification levels. Distances and areas of intimal and medial layer were calculated by digital imaging software (Image J, NIH) using arbitrary parameters. **a** Intimal (I) and media (M) surface area compared in WT ($n=6$) and KO ($n=6$) carotid arteries. KO intimal surface area is significantly less than WT ($*P<0.05$). There was no statistically significant difference between WT and KO media surface area ($P=0.09$). **b** Neointimal formation shown by I/M ratio was shown to be significantly decreased in *Cd39* null mice at day 21 following injury ($*P<0.05$)

Fig. 5 Proliferation assay in wild type and *Cd39*-null VSMC **a**: Proliferative response (thymidine incorporation) to serum and PDGF stimulation. Primary mouse VSMCs from control and *Cd39*-null mice were seeded on 96-well plates (1×10^4 cells/well) and subsequently serum-starved (0.2% FCS) for the following 24 h and then stimulated with 10% FCS containing medium, 50 nM PDGF for 24 h. Twelve hours prior to harvest, cells were pulsed with [3 H]-thymidine. Columns, average of thymidine incorporated count ratio to controls, for three experiments (in triplicate for each experiment), bars, SE. $*P<0.05$. **b** Proliferative response to different concentrations of ATP. Conditions are similar to prior study, except pulse stimulation with ATP was for 5 min; cells were kept in 0.2% FCS for completion of 24 h. Columns, average of thymidine incorporated count ratio to controls, for three experiments (in triplicate for each experiment), bars SE. $*P<0.05$

Cd39-null VSMC show aberrant proliferative responses

To determine differential rates of proliferative responses in vitro, WT and *Cd39*-null VSMC were stimulated with media containing 10% serum and/or PDGF. Paradoxically, the *Cd39*-null VSMC exhibited pronounced proliferative responses to both serum and PDGF stimulation. Exogenous serum increased thymidine incorporation 4.5 ± 0.4 -fold in WT vs. 7.3 ± 0.8 -fold increase in *Cd39*-null ($P<0.05$). PDGF increased thymidine incorporation 6.3 ± 0.7 -fold in WT vs. 8.3 ± 1.1 -fold increase in *Cd39*-null ($P<0.05$; Fig. 5a).

Cd39-null VSMC were minimally responsive to stimulation with different concentrations of ATP when compared to WT VSMC (Fig. 5b). The differential

responses of wild type and *Cd39-null* VSMC were noted when cells were stimulated with 10 and 100 μ M concentrations of ATP.

We noted a 1.47 ± 0.17 -fold increase in wild type vs. 0.88 ± 0.12 -fold increase in *Cd39-null* VSMC after stimulation with 10 μ M ATP and 1.8 ± 0.42 -fold increase in wild-type vs. 0.88 ± 0.12 -fold increase in *Cd39-null* VSMC after stimulation with 100 μ M ATP. Exposure to ATP for more than 5 min decreased cellular proliferation of both WT and *Cd39-null* VSMC, as observed following exposure to ADP and other nucleotides (data not shown).

Cd39-null VSMC show impaired chemoattractant properties

The chemotactic effects of ATP, UTP and PDGF were studied in cultured WT and *Cd39-null* VSMC, using Transwell systems. We demonstrated that *Cd39-null* cells fail to migrate in response to ATP (100 μ M), UTP (100 μ M) and PDGF (50 nM; all have $P < 0.005$; Fig. 6). These decreased levels of migration in the *Cd39 null* VSMC were found to be comparable to the published findings in mutant monocytes [20].

Discussion

Our data indicate that *Cd39* is the dominant ectonucleotidase in VSMC and that extracellular nucleotide signaling plays an important role in VSMC functions. Deletion of *Cd39* in VSMC profoundly decreases cell-associated NTPDase-specific activity. Minimal preservation of VSMC ATPase activity in the *Cd39-null* environment might be

explained by the presence of other ENTPD/*Cd39* family members, including *Cd39L1*.

This is the first study to confirm *Cd39* expression in VSMC in vitro and demonstrate a putative role for this ectoenzyme in the regulation of cellular functions. In particular, we show that deletion of *Cd39* impairs migration of VSMC in response to nucleotides or growth factors in vitro and appears to ameliorate neointimal formation in injured carotid arteries in vivo.

Impaired migration of *Cd39-null* VSMC in vitro might explain the observed decrease in neointimal formation after vascular injury. Indeed, we have previously shown impaired migration and adhesion of *Cd39-null* monocytes and microglial cells upon extracellular matrix proteins [20, 26]. These data also show that *Cd39 null* microglial cell migration can be corrected by dual stimulation with ATP and adenosine [26]. Similar effects of ATP and adenosine on leukocyte migration have been described in neutrophils [27].

This migration abnormality has been shown to be associated with desensitization of nucleotide receptor P2Y-mediated signaling pathways in the setting of extracellular nucleotide excess [20]. Published data in Goepfert et al., suggested that P2Y₁- and P2Y₂-receptor desensitization responses after deletion of *Cd39*, may explain disordered migration of *Cd39-null* macrophages. Furthermore, Weisman et al. have shown that in vivo up regulation of P2Y₂ receptor mRNA in medial VSMC initiates neointimal formation. These P2Y₂-mediated effects of nucleotides have been shown to tightly regulate and modulate VSMC function [28, 29].

Another mechanism to globally inhibit P2Y-mediated responses would be to scavenge extracellular nucleotides by high-level expression patterns of *Cd39*. Furukoji et al., have shown that expression of placental derived CD39 in rat carotid arteries suppresses thrombus formation and also suggest that subsequent neointimal formation may be limited by this manipulation [30]. Our own studies with rat aortic intimal injury suggest that human vascular CD39 up regulation with adenoviral vectors has comparable protective effects [31].

These somewhat puzzling effects may be explained by the desensitization of select P2Y receptors that in the absence of NTPDase activity has the same effect on receptor-signaling events, as would excessive scavenging of nucleotide, when CD39 is upregulated [30, 31]. Hence, both knock-out mice null for *Cd39* and transgenic mice over expressing CD39 have prolonged bleeding times secondary to platelet dysfunction, predominantly at the P2Y₁ receptor level albeit at different pericellular nucleotide levels [21, 32]. We consider the *Cd39-null* VSMC defects, as shown here, to have a comparable underlying cause related to P2Y-type desensitization.

Fig. 6 Impaired migration of *Cd39-null* VSMC. Transwell systems were used to assess the chemotactic effects of PDGF, ATP, and UTP on cultured WT and *Cd39-null* VSMC. In these experiments, 5×10^6 cells were placed in each well. In the lower chamber, the medium consisted of basal media with 0.5% serum (used as control), and basal media with PDGF, ATP, and UTP (see text). Cells migrating to the lower surface of the filter within 6 h were counted. Columns show averages of cells for three experiments (in triplicate for each experiment), counted per 5 HPF/well, and indicating migratory responses; bars denote SE. * $P < 0.005$

In conclusion, we demonstrate the key role and importance of Cd39 expression in the VSMC ectonucleotidase activity and its effect on modulation of associated cellular functions. Moreover, Cd39 deletion impairs VSMC migration in vitro and inhibits neointimal formation in a murine model of injured carotid arteries. Our data provide important insights for the development of novel therapeutic strategies targeted at progressive atherosclerotic diseases and allograft vasculopathy.

References

- Ellsworth ML, Forrester T, Ellis CG, Dietrich HH (1995) The erythrocyte as a regulator of vascular tone. *Am J Physiol* 269: H2155–H2161
- Hamada K, Takuwa N, Yokoyama K, Takuwa Y (1998) Stretch activates Jun N-terminal kinase/stress-activated protein kinase in vascular smooth muscle cells through mechanisms involving autocrine ATP stimulation of purinoceptors. *J Biol Chem* 273:6334–6340. doi:10.1074/jbc.273.11.6334
- Lazarowski ER, Homolya L, Boucher RC, Harden TK (1997) Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem* 272:24348–24354. doi:10.1074/jbc.272.39.24348
- Coad SB, Pearson JD (1989) Metabolism of adenine nucleotides in human blood. *Circ Res* 65:531–537
- Luthje J (1997) Origin, metabolism and function of extracellular adenine nucleotides in the blood. *Klin Wochenschr* 67:317–327. doi:10.1007/BF01741386
- Wang DJ, Huang NN, Heppel LA (1992) Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. *J Cell Physiol* 153:221–233. doi:10.1002/jcp.1041530202
- Malam-Souley R, Campan M, Gadeau AP, Desgranges C (1993) Exogenous ATP induces a limited cell cycle progression of arterial smooth muscle cells. *Am J Physiol* 264:C783–C788
- Erlinge D, Yoo H, Edvinsson L, Reis DJ, Wahlestedt C (1993) Mitogenic effects of ATP on vascular smooth muscle cells vs. other growth factors and sympathetic cotransmitters. *Am J Physiol* 265:H1089–H1097
- Erlinge D, You J, Wahlestedt C, Edvinsson L (1995) Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol* 289:135–149. doi:10.1016/0922-4106(95)90178-7
- Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C (1996) Nucleotide receptor P2_u partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol* 166:57–65. doi:10.1002/(SICI)1097-4652(199601)166:1<57::AID-JCP7>3.0.CO;2-F
- Harper S, Webb TE, Charlton SJ, Ng LL, Boarder MR (1998) Evidence that P2Y₄ nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br J Pharmacol* 124:703–710. doi:10.1038/sj.bjp.0701895
- Plesner L (1995) Ecto-ATPases: identities and functions. *Int Rev Cytol* 158:141–214. doi:10.1016/S0074-7696(08)62487-0
- Cote YP, Picher M, St-Jean P, Beliveau R, Potier M, Beaudoin AR (1991) Identification and localization of ATP-diphosphohydrolase (apyrase) in bovine aorta: relevance to vascular tone and platelet aggregation. *Biochim Biophys Acta* 1078:187–191
- Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC (1996) Identification and characterization of Cd39/vascular ATP diphosphohydrolase. *J Biol Chem* 271:33116–33122. doi:10.1074/jbc.271.51.33116
- Kittel A, Kaczmarek E, Sevigny J, Lengyl K, Csizmadia E, Robson SC (1999) Cd39 as a caveolar-associated ectonucleotidase. *Biochem Biophys Res Commun* 262:596–599. doi:10.1006/bbrc.1999.1254
- Marcus AJ, Hajjar KA, Ullman HL, Islam N, Broekman MJ, Eiroa AM (1991) Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. *J Clin Invest* 88:1690–1696. doi:10.1172/JCI115485
- Marcus AJ, Safier LB, Broekman MJ, Islam N, Fliessbach JH, Hajjar KA, Kaminski WE, Jendraschak E, Silverstein RL, von Schacky C (1995) Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions. *Thromb Haemost* 74:213–217
- Robson SC, Candinas D, Siegel JB, Kopp C, Millan M, Hancock WW, Bach FH (1996) Potential mechanism of abnormal thromboregulation in xenograft rejection-loss of ectoATPases upon endothelial cell activation. *Transplant Proc* 28:536
- Candinas D, Koyamada N, Miyatake T, Siegel J, Hancock WW, Bach FH, Robson SC (1996) Loss of rat glomerular ATP diphosphohydrolase activity during reperfusion injury is associated with oxidative stress reactions. *Thromb Haemost* 76:807–812
- Goepfert C, Sundberg C, Sevigny J, Enjoi K, Hoshi T, Csizmadia E, Robson SC (2001) Disordered cellular migration and angiogenesis in cd-39 null mice. *Circulation* 104:3109–3115. doi:10.1161/hc5001.100663
- Enjoi K, Sévigny J, Lin Y, Frenette PS, Christie PD, Esch JS 2nd, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC, Rosenberg RD (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med* 5:1010–1017. doi:10.1038/12447
- Manka DR, Wiegman P, Din S, Sanders JM, Green SA, Gimple LW, Ragosta M, Powers ER, Ley K, Sarembock IJ (1999) Arterial injury increases expression of inflammatory adhesion molecules in the carotid arteries of apolipoprotein-E-deficient mice. *J Vasc Res* 36:372–378. doi:10.1159/000025676
- Sevigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, Imai M, Zimmermann H, Robson SC (2002) Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. *Blood* 99:2801–2809. doi:10.1182/blood.V99.8.2801
- Chamley-Campbell J, Campbell GR, Ross R (1979) The smooth muscle cell in culture. *Physiol Rev* 59:1–61
- Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca²⁺-stimulated ATPase activity. *Anal Biochem* 157:375–380. doi:10.1016/0003-2697(86)90640-8
- Färber K, Markworth S, Pannasch U, Nolte C, Prinz V, Kronenberg G, Gertz K, Endres M, Bechmann I, Enjoi K, Robson SC, Kettenmann H (2008) The ectonucleotidase Cd39/ENTPDase1 modulates purinergic-mediated microglial migration. *Glia* 56:331–341. doi:10.1002/glia.20606
- Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG (2006) ATP release guides neutrophil chemotaxis via P2Y₂ and A₃ receptors. *Science* 314:1792–1795. doi:10.1126/science.1132559
- Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, Gonzalez FA, Weisman GA (2002) Functional P2Y₂ nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* 106:2720–2726. doi:10.1161/01.CIR.0000038111.00518.35
- Seye CI, Yu N, Jain R, Kong Q, Minor T, Newton J, Erb L, Gonzalez FA, Weisman GA (2003) The P2Y₂ nucleotide receptor

- mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J Biol Chem* 278:24960–24965. doi:[10.1074/jbc.M301439200](https://doi.org/10.1074/jbc.M301439200)
30. Furukoji E, Matsumoto M, Yamashita A, Yagi H, Sakurai Y, Marutsuka K, Hatakeyama K, Morishita K, Fujimura Y, Tamura S, Asada Y (2005) Adenovirus-mediated transfer of human placental ectonucleoside triphosphate diphosphohydrolase to vascular smooth muscle cells suppresses platelet aggregation in vitro and arterial thrombus formation in vivo. *Circulation* 111:808–815. doi:[10.1161/01.CIR.0000155239.46511.79](https://doi.org/10.1161/01.CIR.0000155239.46511.79)
31. Koziak KBM, Robson SC, Bojakowski K, Soin J, Csizmadia E, Religa P, Gaciong Z, Kaczmarek E (2008) Overexpression of CD39/nucleoside triphosphate diphosphohydrolase-1 decreases smooth muscle cell proliferation and prevents neointima formation after angioplasty. *J Thromb Haemost* 6:1191–1197. doi:[10.1111/j.1538-7836.2008.03019.x](https://doi.org/10.1111/j.1538-7836.2008.03019.x)
32. Dwyer KM, Robson SC, Nandurkar HH, Campbell DJ, Gock H, Murray-Segal LJ, Fisicaro N, Mysore TB, Kaczmarek E, Cowan PJ, d'Apice AJ (2004) Thromboregulatory manifestations in human CD39 transgenic mice and the implications for thrombotic disease and transplantation. *J Clin Invest* 113:1440–1446