Extracellular Ribonucleic Acids (RNA) Enter the Stage in Cardiovascular Disease
Alma Zernecke, Klaus T. Preissner

Abstract: Inflammatory and ischemic cardiovascular diseases, especially atherosclerosis and myocardial infarction, remain the number one cause of death in the Western world, whereas the therapeutic options currently available are still limited. Several recent findings have indicated that nucleic acids, particularly extracellular ribosomal RNA and micro-RNAs, significantly contribute to the adverse outcome of atherosclerosis, myocardial infarction, and other cardiovascular diseases. Extracellular RNAs act as novel danger-associated molecular pattern signals and potent cofactors in cardiovascular inflammation and thrombosis, particularly when accumulating in the extracellular space under tissue-damaging or pathological conditions. In this concise review article, the different entities of extracellular RNAs, their cellular sources, and their putative functional contribution to the pathogenesis of cardiovascular diseases will be discussed. In fact, it remains a tightrope walk for these polyanionic molecules outside cells to promote defense reactions on the one side but to provoke cardiovascular disease development on the other side, dependent on their concentration, the environmental conditions, and the cellular stimuli engaged. Thus, we will discuss the mechanisms and cellular responses by which extracellular RNAs operate between defense and disease. Finally, natural counteracting molecules, such as RNase1, will be focused on to elaborate their protective functions in the context of inflammatory and ischemic cardiovascular diseases with the possibility to apply them as novel interventional strategies. (Circ Res. 2016;118:469-479. DOI: 10.1161/CIRCRESAHA.115.307961.)

Key Words: atherosclerosis ■ inflammation ■ myocardial infarction ■ reperfusion injury ■ thrombosis

Cell-Derived Alarmins in Health and Disease
During tissue injury and on exposure of tissue toward stress conditions, such as oxygen radicals, microbial pathogens, hypoxia, or hyperthermia, large amounts of intracellular components are released that disseminate into the circulation or remain at the site of cell activation/damage. Many of these molecules may activate signaling events that contribute to defense and alarm reactions in relation to inflammation or host protection, but some alarmins, particularly histones, may also provoke cytotoxicity. These diverse alarmins (also called danger-associated molecular patterns [DAMPs]), including cytosolic, mitochondrial, or nuclear proteins, as well as nucleic acids, not only play important functions inside cells in the absence of injury but also fulfill multitasking jobs outside cells as moonlighting factors. In particular, nuclear proteins, such as histones, heat-shock proteins, or amphoterin, as well as nuclear DNA, rRNA, and micro-RNAs (miRNAs), become liberated in isolated or complexed form, such as in exosomes or microvesicles. Alarmins may promote immediate sensitization of the innate immune system via activation of, for example, pattern recognition receptors, such as Toll-like receptors (TLR), resulting in an acute inflammatory response and its eventual resolution. Moreover, alarmins, particularly in concert with microbial factors (also indicated as pathogen-associated molecular patterns), play an important role in (chronic)inflammatory disorders, including cardiovascular diseases. As such, alarmins may serve as potential diagnostic or prognostic biomarkers and provide interesting new therapeutic targets for interventional strategies.1

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Emerging evidence suggests an important role of eRNA also in cardiovascular disease. In the following, the putative (patho)physiological functions of high-molecular weight forms of eRNA will be highlighted and discussed in the context of possible therapeutic interventions in cardiovascular diseases. It should be noted, however, that in many cases, we currently do not yet understand whether certain types of eRNA, structural prerequisites, or specific nucleotide sequences of the RNA are required to exert their functions, which is an uncertainty embodied by the rather unspecific term eRNA. The function of miRNAs in this context has been reviewed elsewhere\cite{12-15} and will not be discussed in detail.

**Self-eRNA**

Low levels of eRNA (<100 ng/mL), representing (in the order of descending concentration) rRNA, long noncoding RNA, miRNAs, and mRNA, can be detected in extracellular fluids or in association with cells or tissue, such as spleen sinuses,\cite{16} under quiescent conditions in vivo and in vitro.\cite{11,17} Under conditions of cell activation, trauma or pathology, such as hypoxia, infection, inflammation, or tumor growth, the concentration of undegraded self-eRNA in body fluids can increase dramatically.\cite{18} Increased levels of circulating eRNA are for instance detected not only in patients with tumors, sepsis, or lung fibrosis\cite{17,18} but also after arterial injury, in atherosclerosis-prone mice with myocardial infarction and ischemia–reperfusion (I/R) injury.\cite{20}

The proportion of different forms of released eRNA seems to correspond to the quantities of RNA species found inside cells, whereas the liberation of certain miRNAs has been linked to the cell type–specific generation of exosomes or microvesicles. Although not systematically investigated yet, the entity of released eRNA, its composition, and its potential association with microvesicles largely depend on the investigated cell type, the particular stimulus or type of cell injury, and the (patho)physiological context. For instance, elevated levels of eRNA in plasma collected from perfusates during I/R of isolated rat hearts in response to hypoxia were primarily associated with microvesicles, whereas the majority of eRNA in unstimulated cell-free plasma represented the typical 28S/18S pattern of rRNA.\cite{18,20} Likewise, damage of cells and unregulated cell death result in (passive) liberation of appreciable amounts of RNA as the predominant RNA species.\cite{21} In addition, long noncoding RNAs confer interactions outside and inside cells,\cite{22} and functional and signaling properties of such high-molecular weight RNA species in vascular cells were recently uncovered in part by our group.\cite{18,23}

**Microvesicle-Associated and Complexed eRNA**

Although usually degraded by RNases, precluding its use as a reliable and stable biomarker, the existence of increased, stabilized eRNA under pathological conditions suggests its protection against degradation by binding to proteins and phospholipids\cite{19} or by associating with DNA in extracellular chromatin complexes.\cite{24} Appreciable amounts of eRNA also become associated with the extracellular matrix, where they may bind to fibrillar collagens, von Willebrand factor, or the fibrin clot.\cite{25} Direct interactions with a variety of extracellular proteins may provide eRNA with as yet unrecognized

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### Extracellular Nucleic Acids

More than 50 years ago, endogenous nucleic acids were identified in extracellular fluids and cell supernatants,\cite{2} and the appearance of self-DNA in blood was associated with tissue injury and some diseases, such as systemic lupus erythematosus.\cite{3} Only during the past decade, however, a comprehensive analysis of various forms of extracellular DNA\cite{4} provided unexpected insight into their properties as multifunctional alarmins: these include neutrophil extracellular traps (NETs), which represent the entire nucleosome material of neutrophils that is ejected as ultralarge DNA–histone scaffolds to catch and kill microbial invaders in the context of innate immunity and host defense. NETs in addition promote the activation of the extrinsic and intrinsic pathways of blood coagulation that culminate in fibrin formation, and these prothrombotic properties add to the protective role of NETs in the prevention of microbial dissemination, coined immunothrombosis. However, if not counteracted properly, NETs also promote the development of arterial and venous thrombosis, as demonstrated in preclinical models.\cite{4,5} Among authentic clinical samples, arterial thrombi contain the highest density of NETs and are prone to thrombolysis by mixtures of DNase and tissue-type plasminogen activator.\cite{6} In addition, extracellular mitochondrial DNA, derived from cell injury and resembling CpG-containing bacterial DNA, is recognized as a DAMP by TLR9 to foster sterile inflammatory responses.\cite{7}

Also different forms of extracellular RNA (eRNA), derived from activated or injured cells, are detectable in body fluids and in association with extracellular tissue sites. Considering the amount of RNA within tissue, for example, rabbit tissue contains ≈3 mg of total RNA per gram of tissue,\cite{8} micromolar concentrations of eRNA may be released from injured tissue. In patients with cancer, free eRNA and RNA-proteolipid complexes were regarded as specific secretory products of cancer cells,\cite{9,10} and the detection of circulating mRNA in these patients proved to be useful as a potential biomarker in tumor diagnosis.\cite{11}

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### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
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<tr>
<td>eRNA</td>
<td>extracellular RNA</td>
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<tr>
<td>FSAP</td>
<td>factor VII–activating protease</td>
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<tr>
<td>HFD</td>
<td>high-fat diet</td>
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<tr>
<td>I/R</td>
<td>ischemia–reperfusion</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>miRNA</td>
<td>micro-RNA</td>
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<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
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<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>RIPC</td>
<td>remote ischemia-preconditioning</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>TACE</td>
<td>tumor necrosis factor–α-converting enzyme</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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properties (Table), many of which go beyond the alarming function of eRNA as DAMPs and may provoke cardiovascular pathologies.

eRNA can also be released from cells in association with vesicular structures, as demonstrated for a large number of specific miRNAs or miRNA transcripts, derived from human or mouse tissues or cell lines.\textsuperscript{12–15,32–35} In this regard, miRNAs are the most extensively studied eRNA and may serve at least as qualitative biomarkers for specific pathologies, including cancer, diabetes mellitus, liver injury, or cardiovascular diseases.\textsuperscript{33,36–38} The majority of peripheral blood microvesicles seem to be derived from platelets, whereas mononuclear phagocytes, including macrophages, are the second most abundant source of microvesicles.\textsuperscript{39} Horizontal transfer of microvesicles and subsequent uptake of genetic material by target cells can contribute to alterations in their protein expression patterns and may change during pathological situations, immune responses, or tumor growth.\textsuperscript{36,38,40–43} For instance, macrophage-derived microvesicles contain miRNA that controls differentiation in monocytes and can be translocated to various types of target cells.\textsuperscript{39} Notably, endothelial apoptotic microvesicles contain a miRNA signature that is distinct from the intracellular miRNA composition of the donor cells,\textsuperscript{34} indicating a selective export or packaging of eRNA cargo.\textsuperscript{37,44} The mechanisms of microvesicle shedding and the characteristics of their cellular uptake remain largely obscure.

miRNA in blood can also circulate devoid of lipid microvesicles in ribonucleoprotein complexes, and a key effector protein of miRNA-mediated silencing, argonaute 2, was identified in association with such circulating miRNAs.\textsuperscript{45} Moreover, small RNA species (15–30 nucleotides in length) can be found in association with high-density lipoproteins in plasma, and this transport phenomenon may contribute to the differences of miRNA profiles under normal and atherogenic conditions.\textsuperscript{46} Whether such alliances also apply to other eRNA species remains to be elucidated.

In the following, we focus on the emerging concept that eRNAs enter the stage as new extracellular players in cardiovascular disease.

### eRNA in Cardiovascular Diseases

Atherosclerosis is the main cause of ischemic diseases, such as myocardial infarction and stroke, and is recognized as a chronic inflammatory disease of the arterial wall. Recruitment of circulating blood leukocytes to the inflamed vascular intima and the injured myocardium, local inflammation, and prothrombotic processes are critical determinants of atherosclerotic plaque growth and myocardial remodeling.\textsuperscript{47,48}

A contribution of different eRNA species is emerging as critical mediators of these mechanisms that control (ischemic) cardiovascular disease. As recent reviews have specifically concentrated on various aspects of miRNAs in this context,\textsuperscript{12–15} these issues will not be dealt with here in detail.

#### Role of eRNA in Atherosclerosis

Vascular degeneration in the context of atherosclerosis starts early in life and involves, among others, the shear stress-related disturbance of vascular endothelial cell functions at particular predilection sites (such as bifurcations).\textsuperscript{37} Although endothelial cells exposed to turbulent flow/low shear stress seem to develop a proinflammatory phenotype, high laminar flow/shear stress induces an anti-inflammatory, atheroprotective cell phenotype. Both situations significantly differ by the upregulation and downregulation, as well as shedding of particular endothelial miRNAs that markedly influence posttranscriptional processes in accordance with the observed phenotype switch.\textsuperscript{49,50} Moreover and as further discussed elsewhere,\textsuperscript{12–15} circulating levels of several miRNAs were shown to be significantly altered in patients with coronary artery disease and may serve as biomarkers of disease.\textsuperscript{33,51} A release of other species of eRNA under these conditions, in particular rRNA, and their potential suitability as predictors of cardiovascular disease have not been addressed yet.

In contrast, the tissue distribution and potential activity of eRNA, presumably the most prevalent rRNA type, within atherosclerotic lesions have recently been recognized (Figure): although only trace amounts of eRNA were detected in healthy blood vessels by confocal microscopy with the help of an RNA-binding fluorescent dye, a time-dependent accumulation of eRNA was discernable at later stages within the intima and media of low-density lipoprotein receptor–deficient (\textit{Ldlr}\textsuperscript{−/−}) mice fed a high-fat diet (HFD), particularly in the atherosclerotic central core region and in the vicinity of macrophages in advanced atherosclerotic lesions.\textsuperscript{50} Accumulated eRNA in atherosclerotic plaques may not only be derived from damaged or apoptotic cells but also be released on inflammatory cell activation, as supported by findings showing that tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) stimulation of smooth muscle cells (SMCs) provoked the release of eRNA in vitro.\textsuperscript{50} The exact nature of eRNA species deposited under these conditions remains to be defined. In addition, the conditions and stimuli triggering the release of eRNA in atherosclerosis will need to be addressed in further detail in future studies as well.

Such released eRNA in atherosclerotic lesions may unfurl significant damaging activity together with macrophage-derived cytokines in atherogenesis,\textsuperscript{52} as revealed by

### Table. Binding Proteins for eRNA in the Vascular System

<table>
<thead>
<tr>
<th>Protein</th>
<th>Functional Context</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Vascular endothelial growth factor-A</td>
<td>eRNA serves as cytokine cofactor, induction of hyperpermeability</td>
<td>26,27</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>eRNA forms multimeric complexes, induction of autoimmunity</td>
<td>28</td>
</tr>
<tr>
<td>Coagulation factors XII, XI, prekallikrein, and high-molecular weight kininogen</td>
<td>eRNA serves as autoactivation cofactor to promote the initiation of intrinsic blood coagulation</td>
<td>25</td>
</tr>
<tr>
<td>Factor VII–activating protease</td>
<td>eRNA serves as autoactivation cofactor of this serine protease</td>
<td>29</td>
</tr>
<tr>
<td>HRG</td>
<td>HRG neutralizes the procoagulant function of eRNA</td>
<td>30</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>eRNA stabilizes the serine protease inhibitor for inactivation of target enzymes</td>
<td>31</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Decoration of thrombus</td>
<td>25</td>
</tr>
</tbody>
</table>

eRNA indicates extracellular RNA; and HRG, histidine-rich glycoprotein.
experimental degradation of eRNA. In particular, RNase1 administration in HFD-fed Apoe−/− mice after injury not only decreased plasma eRNA levels but also significantly lowered leukocyte recruitment to carotid arteries, diminished vascular macrophage content and inflammation, and reduced neointimal plaque formation. Moreover, the injury-induced upregulation of cytokines, such as Tnf-α, Il-1β, and Il-6, and adhesion molecules, such as Vcam-1, Icam-1, P-selectin, and Cc12, was abrogated in RNase1-treated mice.20 As administered RNase1 unspecifically degrades all available eRNA substrates, it is not possible to draw conclusions about the specific RNA species involved. The potential molecular mechanisms engaged by eRNA underlying these observations are outlined below.

Taken together, eRNA not only serves as a cell-injury marker in the onset and progression of atherosclerosis but also induces the release of cytokines from vascular and inflammatory cells, whereby the liberation of TNF-α bears an additional positive feedback in this cell damaging cycle. These findings furthermore indicate that RNase1 may provide a novel therapeutic agent to interfere with the adverse eRNA–TNF-α interplay.

**eRNA in Myocardial Infarction and I/R Injury**

After myocardial infarction or I/R injury, the recovery and preservation of cardiac function are important therapeutic aims to achieve. On the basis of the relationship between the hypoxic microenvironment or tissue ischemia and the cellular liberation of eRNA and cytokines, experimental models of I/R injury (mouse and isolated rat heart) were used to uncover the role of eRNA herein. Although extracellular rRNA was predominantly derived from injured cardiomyocytes and induced TNF-α shedding, TNF-α in turn promoted further eRNA release especially under hypoxia, feeding a vicious cell damaging cycle during I/R with the massive production of oxygen radicals, mitochondrial obstruction, decrease in antioxidant enzymes, and decline of cardiomyocyte functions.20 The administration of RNase1, as well as of a TNF-α-converting enzyme (TACE) inhibitor (to block shedding of TNF-α), significantly decreased myocardial infarction and lead to the recovery of physiological parameters of heart function. Likewise, transient myocardial ischemia entailed a marked increase in circulating levels of plasma eRNA, including several miRNAs, and RNase1 administration reduced myocardial cytokine responses and attenuated neutrophil and lymphocyte infiltration and proapoptotic caspase-3 expression, associated with a reduced infarct size already 1 day after I/R injury.53 These findings indicate that eRNA, predominantly derived from ischemic myocardium, plays an adverse role in myocardial infarction, whereby RNase1 administration serves as an effective cardioprotective regimen. Given its potentially broad mode of action at various sites of the body, the exact mechanistic role of eRNA and particular RNA species, therefore, remains to be elucidated.

Notably, although ameliorating myocardial infarction, deficiency in TLR3 had no effect on cardiac cytokine production in vivo,53 implying that self-eRNA contributes to inflammatory responses mostly independent of TLR3.54 Similarly, eRNA elicited cytokine responses in bone marrow–derived macrophages independently of TLR3, TLR7, or TLR8.55 In a recent report, however, it was demonstrated that cardiomyocytes responded to eRNA (isolated from cardiomyocytes or from heart tissue) especially in the presence of lipofectamine with

**Figure.** Contribution of extracellular RNAs (eRNAs) to vascular homeostasis and cardiovascular pathologies. After cell stress (such as hypoxia, injury, and pathogen exposure), eRNA is found to be expressed at various extracellular sites, both intravascularly and extravascularly. In addition, eRNA may be derived from damaged or apoptotic cells or becomes released on inflammatory cell activation and accumulates within atherosclerotic plaques in the course of disease development. The following functional activities of eRNA are promoted by direct interactions with specific extracellular proteins: (1) Promotion of vascular hyperpermeability by eRNA as coreceptor/cofactor of vascular endothelial growth factor (VEGF) and by induction of vasculogenesis/angiogenesis. (2) Initiation of intrinsic blood coagulation (via the contact phase proteins) and thrombus formation. (3) Induction of recruitment of leukocytes to the inflamed vessel wall by eRNA-mediated elevation of expression of intercellular adhesion molecule-1 in endothelial cells or vascular cell adhesion molecule-1 and P-selectin in vascular smooth muscle cells. (4) Skewing of monocytes/macrophages toward the M1 proinflammatory phenotype, accompanied by an upregulated expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12, inducible nitric oxide synthase (iNOS), and response factors, such as chemokines. Here, eRNA triggers TNF-α–converting enzyme (TACE) to liberate the functionally active TNF-α. (5) Promotion of cardiomyocyte death by inducing a hyperinflammatory cascade involving TNF-α in the context of ischemia–reperfusion injury.
the induction of inflammatory factors in a TLR7–MyD88–dependent manner.56 Because these data were derived with the help of transfection agents, it remains to be established in which way eRNA can naturally gain access to endosomal TLR7 in the absence of such chemicals.

### Vascular Functions of eRNA

As established in in vitro and in vivo experimental models, eRNA has many different functions and can directly act on the vessel wall, inflammatory cells, or the myocardium, thereby serving as a cellular stimulator, as a protein cofactor, or as a template for enzyme activation and action.

#### Effect of eRNA on Vascular Permeability

The vascular endothelium is anatomically positioned as a selective barrier between the blood stream and underlying tissues. After the initiation of the innate immune system or vascular injury, various DAMPs can stimulate the quiescent/stable endothelium to release the contents of its Weibel–Palade granules and to express a variety of stress-related factors required for effective vascular response and protection. One important function elicited by eRNA is the induction of a robust and immediate rise in intracellular calcium ions in endothelial cells, associated with an increase in vascular permeability.57,58

Vascular endothelial growth factor (VEGF) serves as a primary multifunctional endothelial permeability and growth factor, promoting several signal transduction pathways to induce angiogenesis, wound repair, and blood vessel regeneration.59 eRNA can directly interact with VEGF to increase the mobilization and local concentration of this cytokine, thereby provoking VEGF-mediated signal transduction via VEGF receptor-2 and neuropilin-1.26,27 eRNA, thus, serves as a coreceptor for VEGF-dependent signaling, reminiscent of the function of cell surface heparin sulfate proteoglycans.27 eRNA-induced hyperpermeability correlates with the disorganization of tight junction proteins (claudin-5 and occludin) and of cytoskeleton-associated proteins (zonula occludens 1 and 2) and a dislodge-ment of VE-cadherin from its cell–cell contact junctions.18

eRNA may furthermore promote vascular permeability on its strong cytokine-inducing activity on monocytes/macrophages. Besides such indirect, cytokine-mediated effects on the endothelium, direct activation of endothelial TLR3 does not play a dominant role in such eRNA-induced permeability changes,26 although a neutralizing antibody against TLR3 somewhat decreased the eRNA-induced interleukin-6 (IL-6) release, indicative for a partial involvement of TLR3 in endothelial cell activation. Furthermore, eRNA-mediated signaling is independent of nuclear factor-κB translocation but involves activation of phospholipase C and leads to exocytosis of Weibel–Palade bodies with the release of vasoactive substances from endothelial cells.

Although not yet tested in the context of cardiovascular disease in vivo, in preclinical models of vasogenic transient cerebral injury and of venous sinus thrombotic occlusion, eRNA was shown to increase the permeability of the blood–brain barrier leading to the development of edema.27,60 underscoring the potent vasopermeable function of eRNA. Such an enhanced permeability with a subsequent increased entry and deposition of, for example, lipoproteins in the intima may promote atherosclerotic lesion formation as well61 or could contribute to cell dysfunction and death in the myocardium.62 It also remains to be investigated whether eRNA induces such events by alternative mechanisms, such as the generation of the vasodilator peptide bradykinin from high-molecular weight kininogen, because eRNA serves as a potent extracellular cofactor for autoactivation of factor VII–activating protease (FSAP) or factor XII; both enzymes are known to promote limited proteolysis of high-molecular weight kininogen.

#### eRNA as a Stimulatory Factor in Tissue and Vessel Regeneration

We have previously shown that eRNA binds to VEGF and stimulates proliferation of microvascular endothelial cells via VEGF receptor-2 in association with neuropilin-1.26,27 In a recent study, extracellular tRNA and rRNA were found to promote vasculogenesis, angiogenesis, and leukopoiesis of stem cells ex vitro in a mouse embryonic stem cell model,63 suggesting that eRNA serves as a functional link between these closely related processes during vessel (re)generation.64 Mechanistically, such types of eRNA seem to efficiently provoke generation of oxygen radicals via activation of nicotinamide adenine dinucleotide phosphate oxidase and stimulation of VEGF-dependent cell signaling, which is in line with (cardio)vasculogenesis strongly depending on the intracellular redox state of differentiating stem cells.65,66 As extracellular DNA is ineffective in this regard, specific features in addition to the polyanionic nature of tRNA and rRNA seem to provide the driving force for vasculogenesis and leukopoiesis. It will be of interest to decipher if eRNA contributes to leukopoiesis (and possibly monopoiesis) under conditions of hyperlipidemia in atherosclerosis or after myocardial infarction to promote cardiovascular disease development. Despite its overall pathogenic nature in this context, it remains to be investigated whether eRNA can also provide protective impulses by these mechanisms, fostering vessel regeneration and tissue healing.

#### eRNA as a Natural Procoagulant Cofactor in Thrombosis

One of the first studies to reveal a physiological function of RNA outside cells aimed to characterize its contribution in the proteolytic activation of the multifunctional hemostatic enzyme FSAP. Here, eRNA (present in cell supernatants or associated with cell surfaces) served as a natural cofactor for (auto)activation of the proenzyme. Although extracellular DNA was hardly effective as a respective cofactor, rRNA from eukaryotic cells, tRNA from yeast, bacterial RNA from Escherichia coli, RNA from hepatitis C virus, and artificial RNA (polyinosinic:polycytidylic acid [poly-IC]) operated as a RNA from hepatitis C virus, and artificial RNA (polyinosinic:polycytidylic acid [poly-IC]) operated as cofactors of the serine protease. This strongly indicates that certain structural requirements of different RNA species, such as nucleotide composition/sequence or secondary structure, seem to be necessary for eRNA’s specific functions.29 The reaction kinetics for (auto)activation of FSAP indicated a template-type mechanism for eRNA function, provided that sufficiently large polyanionic RNA molecules are available to which substrate and enzyme bind simultaneously. Once activated, FSAP can degrade the protease inhibitor, tissue factor pathway inhibitor, thereby indirectly increasing the
blood coagulation–inducing potential of the tissue factor–factor VIIa complex. High plasma levels of FSAP activity in patients with acute coronary syndrome correlate with risk stratification and adverse outcome, which is in line with a procoagulant nature of eRNA as an FSAP cofactor in cardiovascular disease.

Procoagulant functions of eRNA in blood coagulation and thrombosis were subsequently refined by establishing the role of extracellular nucleic acids as primary inducers/cofactors of contact phase coagulation proteins, including factors XII and XI, as well as prekallikrein and high-molecular weight kininogen. Here, direct interactions of eRNA with these polyanion-binding proteins promoted their (auto)activation and conferred a strong prothrombotic effect in vitro and in vivo, indicating that eRNA provides a natural endogenous foreign surface to promote and amplify thrombin generation and thrombus formation alike.

Of note, also NETs have been shown to act as procoagulant inducers of the contact phase/intrinsic coagulation pathway, predominantly by promoting factor XII activation. Thus, tissue injury–related DAMPs seem to enhance blood clotting (as part of innate immunity and body defense) and thereby promote pathological thrombosis, whereas specific inhibition of factor XII–dependent reactions can limit the adverse thrombotic outcome without affecting physiological hemostasis and wound repair. The circulating anticoagulant plasma protein, histidine-rich glycoprotein, was found to specifically weaken the nucleic acid–driven factor XIIa–dependent reaction. These mechanistic relationships underline the importance of eRNA-provoked prothrombotic processes.

In accordance with these findings, eRNA has been found in association with the occluding thrombus in an arterial thrombosis model in mice, and systemic treatment with RNase1 (but not DNase) not only resulted in appreciable smaller, eRNA-free clots but also prolonged the time to arterial occlusion and lead to earlier recanalization of the occluded artery. Similarly, in a rat model of venous sinus thrombotic occlusion, pretreatment with RNase1 decreased the extent of vessel occlusion after injury and increased the rate of later recanalization. Moreover, RNase1 administration reduced infarct volume in an experimental stroke model after temporary middle cerebral artery occlusion in rats. An elevated eRNA burden also seems to be relevant for the development of myocardial reperfusion injury or atherosclerosis, both situations are known to be promoted by prothrombotic disposition. Procoagulant properties of eRNA may also be expanded to the prothrombotic state of patients with infections or malignancies. Interfering with these eRNA-induced thrombotic properties, RNase1, thus, serves as a versatile anticoagulant and antithrombotic regimen.

eRNA and the Inflammatory Response

On the basis of its alarming functions, eRNA resembles other DAMPs as potent stimulators of cytokine expression and release from inflammatory and vascular cells. Because of its multiple interactions with a range of extracellular proteins, functional features of eRNA are best understood in the context of an endogenous eRNA-induced inflammatory cascade.

eRNA and Leukocyte Recruitment

When injected into a living organism (rabbit and mouse), eRNA not only induces a momentary activation of blood coagulation in blood plasma but also triggers the recruitment of inflammatory cells: administered eRNA promotes the adhesion and extravasation of leukocytes in vivo in the murine cremaster muscle vasculature model that was dependent on intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, as well as the leukocyte β2-integrin Mac-1.

Likewise, exposure of quiescent endothelial cells toward eRNA in vitro resulted in an elevated expression of ICAM-1 (but not of other adhesion receptors, such as vascular cell adhesion molecule-1, platelet-endothelial cell adhesion molecule-1, junctional adhesion molecule-A, E-selectin, or P-selectin), ensuing leukocyte adhesion and transmigration. An increased expression of ICAM-1, vascular cell adhesion molecule-1, P-selectin, and the chemokine (CC-motif) ligand 2 was furthermore demonstrated in vascular SMCs on stimulation by eRNA, contributing to monocyte adhesion to SMCs in vitro and to leukocyte recruitment in carotid arteries after vessel injury in vivo. Conversely, administration of RNase1 in such experimental systems reduced monocyte adhesion to activated SMCs under flow conditions in vitro, prevented leukocyte adhesion and transmigration in vivo, and reduced arterial macrophage accumulation after injury in carotid arteries of apolipoprotein E–deficient (Apoe−/−) mice. Overall, these studies strongly implicate that eRNA plays a major role in promoting leukocyte recruitment under inflammatory conditions, whereby the detailed mechanistic relations remain to be elucidated.

As discussed earlier, eRNA can serve as a coreceptor for VEGF-dependent signaling. In this regard, it is interesting to note that neutralizing antibodies against VEGF or the extracellular domain of neuropilin-1 (the coreceptor of VEGF receptor-2) decreased monocyte adhesion and transmigration in the murine cremaster muscle vasculature in vivo and that VEGF increased monocyte adhesion to shear-induced carotid artery plaques and contributed to atherosclerotic plaque expansion in Apoe−/− mice. In addition to the induction of adhesion molecules and proinflammatory cytokines in macrophages, endothelial cells, or SMCs, eRNA may, thus, also engage VEGF-mediated signaling to promote leukocyte adhesion.

eRNA and Macrophage Polarization

In general, monocytes/macrophages respond to external stimuli with rapid changes in the expression of numerous inflammation-related genes to undergo polarization toward the M1 (proinflammatory) or M2 (anti-inflammatory) phenotype. When exposed toward eRNA in vitro, mouse bone marrow–derived macrophages, differentiated with mouse macrophage colony-stimulating factor, were found to be skewed toward the M1 phenotype. This resulted in upregulated expression of inflammatory markers, such as TNF-α or IL-6, together with IL-12 and inducible NO-synthase, whereas anti-inflammatory genes, such as chitinase-like proteins or macrophage mannose receptor-2 (CD206), were significantly downregulated. Similarly, after the treatment of human peripheral blood monocytes with eRNA, microarray analyses of the whole human genome furthermore revealed an upregulation of 79 genes by at least 4 times, of which were related to signal transduction and 15
genes were associated with inflammatory responses, including various (proatherogenic) cytokines and chemokines, such as chemokine (CC-motif) ligand 3, chemokine (CC-motif) ligand 20, and chemokine (CX3-C motif) ligand 2. These data imply that eRNA seems to be a robust and strong proinflammatory factor in the setting of macrophage polarization (Figure). This notion is furthermore supported by the fact that on treatment with RNase1, a significant reduction in proatherogenic cytokines (TNF-α, IL-1β, and IL-6) was observed in the vessel wall of HFD-fed Apoe−/− mice after injury.20

Interestingly, eRNA-mediated cytokine mobilization was largely independent of TLR-induced signaling, and the eRNA-related cytokine responses in bone marrow-derived macrophages (including the release of TNF-α and activation of nuclear factor-κB signaling) could not be duplicated by the TLR3-agonist poly-IC or single-stranded RNA as agonists for TLR7 or TLR8, respectively.54 In this regard, the eRNA-induced expression and release of TNF-α are of particular interest, which involve the intracellular prolytic processing of the prosheddase TACE (or ADAM17) and cleavage of transmembrane pro-TNF by TACE to liberate the functionally active trimeric TNF-α from macrophages and other cells (Figure). Besides pro-TNF, TACE recognizes and cleaves >70 other unrelated membrane-anchored protein substrates, including proepidermal growth factor, Notch, ICAM-1, VEGF receptor-2, IL-6 receptor, L-selectin, or CD44; all of which seem to be connected to cell stimulatory/inflammatory signals in different biological systems. Thus, it is tempting to speculate that eRNA represents a universal alarm signal/DAMP for triggering a ubiquitously expressed single sheddase to induce a variety of stimulatory factors at various tissue sites and of varying strength in the body to foster an inflammatory response. The mechanistic basis by which eRNA promotes TACE activation remains to be addressed in future studies.

Of note, stimulation of mast cells by a variety of agonists induces the release of eRNA, representing predominantly rRNA.1877 Cell supernatants of degranulated mast cells thereby induce cytokine production and release in monocytes and other inflammatory cells in an eRNA-dependent manner, a mechanism that may in addition amplify inflammatory responses in atherosclerosis52 and could be of relevance to other diseases as well.

**eRNA as a Trigger of Immune Responses**

Multimeric complexes between eRNA and the basic protein platelet factor 4 (chemokine [CC-motif] ligand 4) seem to function as initial autoantigenic trigger for the generation of autoantibodies in heparin-induced thrombocytopenia,28 and autoantibodies against RNA-binding proteins were also found to be associated with neurological disorders, such as pediatric opsoclonus–myoclonus syndrome.76 Because of nucleoside modifications in self-RNA (unlike bacterial and mitochondrial RNAs), the potential activation of TLR3−, TLR7−, or TLR8-expressing dendritic cells by eukaryotic RNA may become suppressed to avoid false alarming responses.79 However, self-eRNA, complexed to autoantibodies against the nucleic acid or nucleoprotein component, can provide the delivery of eRNA to endosomal compartments via FcγRII-mediated endocytosis, as shown for plasmacytoid dendritic cells (pDCs).

Self-RNA, liberated from apoptotic or secondary necrotic cells, also binds to the antimicrobial basic peptide LL37, resulting in condensation of free eRNA into compact structures that are protected against RNase degradation.80,81 These eRNA–LL37 complexes associate with proteoglycans of the cellular glycocalix via ionic interactions and become endocytosed.82 Unlike the isolated components, eRNA–LL37 complexes may then acquire the propensity to bind to TLR7 or TLR8 (recognizing single-stranded RNAs as their natural ligand) in human dendritic cells to induce respective alarm signals in the context of innate immunity.83 Notably, the subset of pDCs expresses high levels of TLR7 and TLR9 (the latter recognizing unmethylated CpG sequences in DNA). However, similar to self-RNA as described above, also DNA released from necrotic or apoptotic cells (such as in NETs or contained within circulating immune complexes) can bind TLR9 to activate pDCs in the presence of antimicrobial peptides.84

These mechanisms may also be important in atherosclerosis, given the accumulation of apoptotic/necrotic cells within plaques and the presence of antimicrobial peptides, NETs, or immune complexes within plaques.85 Such contentions are exemplified by findings showing that infusion of complexes of antimicrobial peptides and self-DNA mediated pDC activation and could pDC dependently promote atherosclerotic lesion development in Apoe−/− mice, whereas pDC depletion reduced atherosclerosis.84,85 Moreover, mitochondrial DNA in conjunction with antimicrobial peptide leads to activation of TLR9-mediated inflammation and atherosclerotic lesion formation in Apoe−/− mice.86

Given the conflicting data on the role of both TLR7 and TLR9, for example, in vascular inflammation, it currently remains unclear whether effects of eRNA (and extracellular DNA) on atherosclerosis and myocardial infarction are in part mediated by triggering immune responses targeted against self-nucleic acids that require signaling via particular TLRs. It is conceivable that eRNA exerts cell type–specific mechanisms and that the multiple cardiovascular functions of self-eRNA are mainly transmitted independently of TLR signaling.

**Nucleases as Natural Antagonists of Extracellular Nucleic Acids**

Although eRNA can trigger and propagate multiple cardiovascular functions, its activity can be counteracted or limited by RNase1 administration, which was demonstrated to provide protection in many pathogenetic scenarios. It should be noted that exogenously administered RNase1 does not discriminate in its action between different species of eRNA substrates such that any effect of RNase1 is related to the degradation of available (free) eRNA species. A balance in the eRNA/RNase system is, thus, required to control vascular homeostasis by regulating the extracellular functions of different forms of eRNA and to promote protection of blood vessels and tissues.

**eRNAs as Natural Counterparts of Extracellular Nucleic Acids**

RNase 1 is a member of the eRNaseA superfamily, representing a secretable, thermostable protein with variations in glycosylation and hence variable sizes of 17 to 26 kDa. Although a high production of the enzyme is observed in (exocrine) pancreas,
testis, ovary, and brain, RNase1 is also prominently expressed in vascular endothelial cells of large and medium vessels, and its constitutive secretion into the blood stream ensures a concentration of 300 to 400 µg/L in blood plasma. In addition, SMCs and tumor cells express RNase1. Another RNase isoform, RNase 5, is expressed in a wide spectrum of cells, foremost by the liver and inflammatory cells; yet, its contribution in the pathogenesis of vascular diseases has hardly been studied. RNase5 circulates in normal human plasma in a concentration range of 250 to 350 µg/L; however, the enzyme does not exhibit substantial ribonuclease activity against standard RNA substrates, and only some minor hydrolytic activity against rRNA is observed. Rather, RNase5, known as angiogenin, is one of the strongest proangiogenic factors and also involved in tumor angiogenesis. It is not known in which way this basic protein may affect extracellular binding and the described functional activities of eRNA, without substantially degrading these polyanions.

In endothelial cells, a portion of synthesized RNase1 (like von Willebrand factor, angiopoietin-2, and IL-8) is stored in Weibel–Palade granules that, on stimulation by vasopressin (in vivo) or by prothrombotic or proinflammatory agents, such as thrombin, VEGF, and eRNA itself, momentarily release their contents by exocytosis. Long-term treatment (for several hours) of endothelial cells with proinflammatory cytokines, such as TNF-α or IL-1β, results in significant downregulation of RNase1 mRNA and RNase1 protein levels, mainly involving epigenetic regulation of histone modifications. This indicates that acute inflammatory conditions promote a temporary RNase1 release to degrade the excess of proinflammatory eRNA, whereas chronic stimulatory conditions lead to a decreased RNase1 production, thereby providing another regulatory aspect of the vascular eRNA/RNase system. In which way extracellular miRNAs are affected by changes in circulating RNase1 concentrations remains to be analyzed.

In line with these observations, plasma RNase activity is subject to alterations in vivo, such as in patients with cancer. Also after acute myocardial infarction, serum RNase activity was described to increase at 1 day, peaking at 3 to 5 days and returning to baseline at 14 weeks after injury, compared with healthy controls. In experimental atherosclerosis in Ldlr⁻/⁻ mice, a biphasic characteristic of RNase activity was observed with a temporary increase during the first 2 weeks, followed by a significant and sustained decrease to ≈20% to 40% of the activity in baseline controls, starting at 4 weeks of HFD feeding throughout the 36 weeks of the observation period. Together with the progressive eRNA accumulation within atherosclerotic lesions, these data imply a causal relationship between a dysbalance of the eRNA/RNase system and the initiation and progression of atherosclerosis. Interestingly enough, RNase1 together with other proteins, such as Jun dimerization protein-2 or tissue inhibitor of metalloproteinases-1, was found to serve as a prognostic biomarker associated with the progression of heart failure in patients with ST-segment–elevation myocardial infarction.

eRNA as a Target for Interventional Therapy in Cardiovascular Diseases

In the experimental setting, application of RNase1 proved to be promising as a novel interventional strategy for the therapy of cardiovascular diseases to effectively antagonize eRNA-mediated activities as an alarm signal and a cell damaging factor. In particular, systemic intravenous treatment with RNase1 was shown to delay thrombus formation in mice, and RNase1 treatment strongly reduced cerebral edema formation and infarction size in acute stroke in rats. In atherosclerosis, RNase1 administration in HFD-fed ApoE⁻/⁻ mice resulted in reduced neointimal plaque formation, diminished monocyte recruitment, and a decrease in vascular inflammation after injury. Similarly, RNase1 treatment significantly attenuated myocardial cytokine production, leukocyte infiltration, and cardiomyocyte apoptosis and conferred cardiac protection against I/R injury. These findings provide important preclinical evidence of the effectiveness of RNase1 as a new therapeutic drug in cardiovascular diseases. Because RNase1 is a thermostable, nontoxic enzyme, it bears features that emphasize a possible safe application as an interventional regimen against atherothrombotic complications.

Beyond such interventional RNase1 application, further consideration should also focus on the role of the natural eRNA/RNase system in patients, particularly with respect to the mobilization of RNase1 from intraendothelial storage granules. It is proposed that application of remote ischemia-reperfusion preconditioning (RIPC) in humans should be associated with an increase in the systemic concentration of RNase1 and a possible better outcome of cardiac operations. RIPC is a noninvasive and virtually cost-free strategy for protecting the heart (and other organs) against acute I/R injury. In fact, in a clinical study with 14 cardiac patients, the level of cardioprotective RNase1 increased on application of RIPC, whereas the concentration of damaging eRNA and TNF-α decreased.55 These findings imply a significant contribution of the RIPC-dependent (endothelial) RNase1 for improving the outcome of cardiac surgery. The exact mechanism of RNase1-induced cardioprotection still remains to be explored.

Conclusions

Several lines of evidence show that eRNA serves as a cell-injury marker in the onset and progression of different diseases. Released in the context of cell activation or injury, eRNA is emerging to provide multiple functions as a moonlighting factor outside cells. Although eRNA can promote tissue and vessel regeneration and may, thus, contribute to tissue healing, it also engages in diverse pathogenic pathways in cardiovascular diseases: eRNA promotes vascular permeability, harbors procoagulant activity to trigger prothrombotic complications, induces the release of proinflammatory mediators from vascular and inflammatory cells, and triggers inflammatory cell recruitment; all functions that to some extent were already shown to drive atherosclerosis and cardiac remodeling after myocardial infarction or I/R injury.

Functional diversity of eRNA may in part be linked to interactions with different binding partners, such as cell surfaces, proteins, phospholipids, or DNA, that remain to be elucidated in detail. For instance, eRNA has been shown to directly bind to VEGF, as well as to different polyanion-binding contact phase coagulation proteins for activation, but it also acts by a template-type mechanism to activate proteases. On the cellular level, TLR3 was shown to play a minor role...
in mediating eRNA-induced cytokine responses. The search for TLR-independent eRNA receptors remains a challenge for future studies.

These diverse functions of eRNA have prompted research on its therapeutic targeting. Indeed, application of RNase1 for eRNA degradation has shown to harbor high potential to ameliorate thrombus formation, cerebral edema formation, and infarction size in acute stroke, I/R injury, and atherosclerosis. eRNA degradation has shown to harbor high potential to ameliorate ischemic heart disease.

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The eRNA/RNase system and its actions in cardiovascular disease may thus, constitute a promising novel therapeutic target to ameliorate the pathological outcome of atherosclerosis and ischemic heart disease.

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Disclosures

None.

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