World Journal of Cardiology

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DOI: 10.4330/wjc.v7.i8.434

World J Cardiol 2015 August 26; 7(8): 434-441
ISSN 1949-8462 (online)

EDITORIAL

Thrombosis: Novel nanomedical concepts of diagnosis and treatment

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Author contributions: Cicha I solely contributed to this paper.

Supported by The EU "NanoAthero" project FP7-NMP-2012-LARGE-6-309820; and by the DFG (CI 162/2-1, SPP1681).

Conflict-of-interest statement: Iwona Cicha has no conflict of interest to disclose.

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Received: January 23, 2015
Peer-review started: January 24, 2015
First decision: March 6, 2015
Revised: March 23, 2015
Accepted: May 16, 2015
Article in press: May 18, 2015
Published online: August 26, 2015

Abstract

Intravascular thrombosis, a critical pathophysiological feature of many cardiovascular disorders, leads to the formation of life-threatening obstructive blood clots within the vessels. Rapid recanalization of occluded vessels is essential for the patients' outcome, but the currently available systemic fibrinolytic therapy is associated with low efficacy and tremendous side effects. Additionally, many patients are ineligible for systemic thrombolytic therapy, either due to delayed admission to the hospital after symptom onset, or because of recent surgery, or bleeding. In order to improve the treatment efficacy and to limit the risk of hemorrhagic complications, both precise imaging of the affected vascular regions, and the localized application of fibrinolytic agents, are required. Recent years have brought about considerable advances in nanomedical approaches to thrombosis. Although these thrombus-targeting imaging agents and nanotherapies are not yet implemented in humans, substantial amount of successful *in vivo* applications have been reported, including animal models of stroke, acute arterial thrombosis, and pulmonary embolism. It is evident that the future progress in diagnosis and treatment of thrombosis will be closely bound with the development of novel nanotechnology-based strategies. This Editorial focuses on the recently reported approaches, which hold a great promise for personalized, disease-targeted treatment and reduced side effects in the patients suffering from this life-threatening condition.

Key words: Thrombosis; Thrombus imaging; Nanomedicine; Targeted nanoparticles; Thrombolytic drug-delivery systems

Core tip: The prevalence of thrombosis, the formation of life-threatening clots obstructing vital blood vessels, continues to rise. Accurate diagnosis and rapid recanalization of an occluded artery is essential to improve outcomes and reduce the mortality in acute myocardial infarction or stroke. The current thrombolytic therapy often fails to diminish the occlusion and is associated with a high rate of hemorrhagic complications. Develop-

ment of directed nanosystems for local thrombolysis, characterized by a strong fibrinolytic effect and low bleeding risk, is therefore one of the most urgent tasks in the prevention and the therapy of acute thrombotic events.

Cicha I. Thrombosis: Novel nanomedical concepts of diagnosis and treatment. *World J Cardiol* 2015; 7(8): 434-441 Available from: URL: http://www.wjgnet.com/1949-8462/full/v7/i8/434.htm DOI: http://dx.doi.org/10.4330/wjc.v7.i8.434

INTRODUCTION

Intravascular thrombosis, the formation of life-threatening obstructive blood clots within the vessels, underlies a number of cardiovascular disorders such as heart attack, ischemic stroke, pulmonary embolism, and deep vein thrombosis[1,2]. Among these, atherothrombotic diseases (ischemic heart disease and stroke) are collectively responsible for 25% of all deaths worldwide[3]. In contrast, the burden of venous thromboembolism (VTE) is not well documented. According to the recent analyses[4], its incidence ranges between 1-2 per 1000 individuals in most of the studies, resembling the frequency of myocardial infarction[5], and the deaths due to VTE are estimated at 300000 per year in the United States[6]. Globally, the prevalence of thrombotic disorders continues to increase, particularly in the developing countries. However, despite significant advances in understanding of the disease mechanisms, which led to the development of more effective anti-thrombotic and thrombolytic drugs[7], the effect of these therapies on the patients' outcomes remains disappointing: According to the published data, less than 25% of high cardiovascular risk patients receiving antiplatelet therapy avoided a fatal thrombotic event[8]. The inherent problem with the conventional antithrombotic approaches is the increased risk of bleeding, as the existing therapeutics destabilize hemostatic processes. Among the most urgent challenges in this field are thus (1) identification of patients at increased risk for thrombosis and precise estimation of individual disease burden, as well as (2) development of safe and effective strategies to prevent thrombotic events and/or rapidly diminish vascular occlusion. These challenges have been intensively addressed by the researchers across the globe, resulting in a number of innovative approaches to diagnosis and treatment of thrombosis, as outlined below.

DIAGNOSIS OF THROMBOTIC DISEASE

Individual burden of thrombosis

In order to improve the diagnosis, risk stratification, and management of thrombotic syndromes, reliable methods of *in vivo* assessment of the thrombotic risk in patients with cardiovascular diseases are needed. Although thrombin is the most important serine protease within the coagulation cascade[9], thus far the diagnostic tests are lacking that are able to rapidly and reliably assess its activity in clinical settings. To address this need, a novel urinary nanomarker assay based on thrombin-sensitive iron oxide nanoparticles was recently developed that allows detection of thrombin activity and thus quantitative estimation of thrombosis burden *in vivo*[10]. The nanomarkers were produced by coupling iron oxide nanoworms with thrombin-cleavable peptides linked to a synthetic reporter system, composed of the protease-resistant peptide, glutamate-fibrinopeptide B, which was modified at the termini with ligands detectable by an immunoassay (fluorescein, or Alexa488, and biotin). In a mouse model of pulmonary embolism induced by thromboplastin[11], the circulating nanomarkers sucessfully accessed the local sites of thrombosis and released the reporters upon cleavage by thrombin. The urinary clearance of these reporters was detectable by ELISA with high sensitivity and significantly correlated with the thrombosis burden estimated by the histochemically analyzed amount of fibrin deposited in the lungs[10]. Given the need of rapid and reliable *in vivo* assessment of the thrombotic burden in cardiovascular patients, this urine analysis-based assay represents a very promising platform for use in clinical practice.

Imaging of thrombosis in vivo

During the thrombotic event, initially smaller clots form larger obstructive thrombi, which require a long time for recanalization, and a high dose of thrombolytic agents, or a high rate of mechanical clot disruption[12]. Both the burden and the localization[13,14] of thrombi are known to affect clinical outcomes and mortality[12,15]. By providing essential information about the size and localization of the thrombi, direct thrombus imaging would have an immense impact on clinical practice: Without a tool for *in vivo* imaging in the clinical settings, individualisation of the thrombolytic therapy is impossible. The recommended fixed dose of intravenous tissue plasminogen activator (tPA; 0.9 mg/kg) is thus insufficient in some patients, resulting in resistance to thrombolysis, or excessive in others, leading to increased risk of hemorrhagic complications.

Intravascular thrombus formation therefore represents a target for novel nanoparticle-based diagnostics. As early as in 2001, thrombus detection *in vivo* by magnetic resonance imaging (MRI) was accomplished in dogs using anti-fibrin monoclonal antibodies conjugated to lipid-encapsulated perfluorocarbon nanoparticles containing gadolinium-chelate[16]. More recently, *ex vivo* optical imaging of atherothrombosis in ApoE-deficient mice fed a high-fat diet was reported with lipopeptide nanoparticles carrying a fluorescently-labeled pentapeptide Cys-Arg-Glu-Lys-Ala (CREKA), which binds to clotted plasma proteins in the blood vessels[17]. Intravascular fibrin detection by MRI in this mouse model was also described by Makowski *et al*[18]

using a commercially available gadolinium-based fibrin-binding peptide EP-2104R. Another fibrin-targeting peptide (GPR, Gly-Pro-Arg) has been utilised in studies by Obermeyer *et al*[19] and McCarthy *et al*[20]. The former group applied bacteriophage MS2 capsids functionalized with GPR peptide on the exterior of each protein shell for fibrin imaging. The GPR-modified capsids were evaluated *in vitro* with regard to the fibrin imaging. Near-infrared fluorophores on the interior surface of the capsids enabled optical detection of their binding to fibrin clots with improved signal-to-background ratio as compared with non-targeted nanoagents[19]. Furthermore, in a mouse model of ferric chloride injury to jugular vein, McCarthy *et al*[20] utilised fluorescently labeled cross-linked iron oxide nanoparticles functionalized with GPR or FXIIIa-targeting peptides to obtain multimodal nanoagents exhibiting either covalent or noncovalent binding to thrombi. These nanosystems allowed *in vivo* detection of thrombus by both MRI and optical imaging modalities.

Apart from fibrin, activated platelets represent an important target for detection of intraluminal thrombi and endothelial activation, a marker of ongoing atherothrombotic disease. Therefore, the development of contrast agents for imaging of P-selectin, expressed by activated platelets, has been the aim of numerous efforts. In particular, the research group of Bachelet *et al*[21] and Manzo-Silberman *et al*[22] developed several nanosystems for *in vivo* P-selectin detection based on polysaccharide fucoidan (a mimic of sialyl Lewis X, the natural ligand of P-selectin) derived from brown seaweed. Radiolabeled fucoidan was demonstrated a suitable P-selectin targeting agent for *in vivo* single-photon emission computed tomography imaging of platelet-rich thrombi in rat models of infective endocarditis and elastase-induced aortic aneurysms, as well as endothelial activation in a model of myocardial ischemia-reperfusion[23]. Very recently, this group tested ultrasmall superparamagnetic iron oxide nanoparticles (USPIO) coated with fucoidan for molecular MRI of intraluminal thrombus: In a rat model of elastase-induced aortic aneurysms, all thrombi detected in MRI by USPIO-fucoidan particles were correlated with P-selectin immunostaining and USPIO detection by electron microscopy, whereas no intraluminal thrombi were detectable upon control USPIO[24]. In a study by Ta *et al*[25], iron oxide nanoparticles were coupled with a single-chain antibody that specifically binds to ligand-induced binding sites (LIBS) on glycoprotein IIb/IIIa (CD41/CD61), the most highly expressed molecules on the surface of activated platelets. LIBS-targeting nanocontructs showed a strong and specific binding to activated platelets *in vitro*, as well as *in vivo* by intravital microscopy and MRI of mouse carotid arteries[25].

Iron oxide nanoparticles allow *in vivo* visualization of thrombi with MRI. However, MRI is rarely the test of choice for the management of patients with acute thrombotic events, due to the time restrictions of clinical management, or contraindications for MRI in some patients. For most clinical decision making involving the administration of tPA, computed tomography (CT) is the current standard[26] due to its speed and convenience. However, non-contrast CT often does not allow a precise assessment of extent and distribution of thromboemboli, because the density of the thrombus is often not much different from that of the surrounding blood. Therefore, efficient and safe contrast agents are needed to achieve the enhancement of thrombus imaging with the clinical CT. Addressing this issue, thrombus detection using microCT has been recently tested in a mouse model of ferric chloride-induced carotid thrombosis utilising glycol chitosan (GC)-gold nanoparticles as contrast agents[27]. The study showed that these nanoparticles became trapped in the blood clots proportionally to thrombotic insult, and allowed the quantitative characterization and serial monitoring of thrombus evolution, embolization, and *in situ* recurrent thrombosis, as well as the assessment of therapeutic efficacy of tPA-induced thrombolysis. Due to a long circulating half-life, GC-gold nanoparticles remained available for entrapment into fibrin matrix for up to 3 wk, allowing repetition or ongoing monitoring of thrombogenesis and thrombolysis with microCT[27].

Collectively, the above-discussed studies demonstrate that nanosystems which target fibrin or activated platelets can enhance the detection and the diagnosis of intravascular thrombi by means of existing imaging modalities. Thus far however, little is known about their safety and efficacy in humans. Provided low toxicity and a good therapeutic index, these nanosystems should improve risk stratification when translated into the clinical practice, and allow personalized therapeutic regimen in thrombosis-related diseases.

PREVENTION AND TREATMENT OF THROMBOSIS

Current therapies

Platelet activation and aggregation are the key processes involved in thrombosis and thromboembolic disorders. The best preventive measures for the thrombotic events in the risk patients are thus antiplatelet or anticoagulation therapy. Although aspirin still plays an essential role in the primary and secondary prevention of atherothrombosis, new generation antithrombotic therapies are rapidly evolving. In combination with aspirin, ADP $P2Y_{12}$ receptor antagonists are used in the management of acute coronary syndromes and percutaneous coronary interventions in order to prevent adverse cardiac events and stent thrombosis[28]. Oral anticoagulation used for the treatment of VTE and for the prevention of emboli in patients with atrial fibrillation has advanced with the use of direct thrombin and factor Xa inhibitors that do not require therapeutic monitoring[29]. Although antiplatelet and anticoagulant therapy is essential for the primary and secondary prevention of atherothrombosis, systemic pre-treatment

with antithrombotic agents is associated with increased risk of hemorrhagic complications after intravenous thrombolysis in patients with acute ischemic stroke[30].

Acute management of stroke focuses on stabilizing the patient and ensuring the maximal reperfusion of the ischemic brain tissue. Hence, intravenous thrombolysis remains the mainstay treatment for acute ischemic stroke: A statistically and clinically significant improvement in outcomes is observed for carefully selected patients treated with tPA within 4.5 h of stroke onset[31]. While no other medication has demonstrated comparable efficacy, tPA remains the only drug for acute ischemic stroke approved by Food and Drug Administration since 1996[32]. However, its use is very limited both by the narrow eligibility and administration window, and by the risk of hemorrhagic complications[33], so called thrombolysis-related symptomatic intracerebral haemorrhage, which occurs in about 6% of patients and is associated with nearly 50% mortality[34,35]. Additionally, in some patients eligible for tPA treatment, the outcome is poor, when occlusion occurs in large arteries (internal carotid artery, middle cerebral artery or basilar artery). For these subgroups of stroke patients, an endovascular (intraarterial) administration route has been developed, but its clinical benefit remains unproven. Randomized controlled clinical trials did not show any added benefit of endovascular treatment over intravenous tPA alone in intravenous tPA-eligible patients, even in patients with persistent large-artery occlusion, nor have these trials provided evidence of clinical benefit in patients who were ineligible for intravenous tPA because of being > 4.5 h from symptom onset[36].

Alternative means of reperfusion, ideally based on individual thrombus burden estimation are therefore needed. For this purpose, thrombus-targeted nanosystems could serve as carriers for direct delivery of therapeutic agents to the occlusive thrombi in order to increase the effective local concentrations of antithrombotic drugs.

Antiplatelet and anticoagulant medications for prevention of thrombosis

Current antiplatelet drugs are only partially effective in preventing thrombus formation and thromboembolic events. Consequently, much interest is drawn both to the discovery of novel antiplatelet medications and to the optimization of the existing ones. The group of Chen *et al*[37] reported the synthesis of novel, self-assembly anti-platelet aggregation peptides containing L-arginine and L-aspartic acid, that were complexed with Cu(Ⅱ) to form stable nanoparticles. In a rat model of thrombus formation, these peptides at 5 μmol/kg achieved anti-thrombotic activity comparable to 110 μmol/kg aspirin, whereas the peptide-Cu(Ⅱ)-nanocomplexes were equally effective in reducing the thrombus weight already at 100-fold lower concentrations (0.05 μmol/kg). More recently, the same group reported a successful approach to overcome the low response to aspirin observed in some patients, which severely decreases its efficacy at the tolerated doses[38]. In that study, aspirin was conjugated to the Arg-Gly-Asp-Val (RGDV) tetrapeptide, resulting in a nano-assembly targeting glycoprotein Ⅱb/Ⅲa, the receptor for RGD peptide on the surface of the activated platelets. *In vitro*, aspirin-RGDV particles inhibited platelet aggregation induced by thrombin or arachidonic acid more effectively than free aspirin. A very strong antithrombotic effect of aspirin-RGDV was also observed in a rat model of thrombosis - whereas aspirin exhibited no antithrombotic activity at 16.7 μmol/kg, aspirin-RGDV significantly and dose-dependently inhibited thrombus formation in the treated rats already at doses of 0.1 and 1 nmol/kg[38]. Targeted delivery of aspirin to thrombus and its local release to activated platelets thus resulted in an extraordinarily potent inhibition of thrombus formation, overcoming the apparent non-response to aspirin. Formation of thrombi was also effectively prevented by novel heparin-conjugated carbon nanocapsules in a mouse model of acute hindlimb thromboembolism[39]. Compared to the injection of heparin alone, those heparin-functionalized carbon nanocapsules displayed superior antithrombotic activity *in vitro* and *in vivo*, representing a promising nanocarrier system for anticoagulant delivery.

Some of the most common cardiovascular interventions, including stent implantation or prosthetic heart valve replacement, are associated with increased risk of thrombosis, necessitating prolonged or even lifelong antiplatelet therapy. Particularly after cessation or premature discontinuation of the therapy, the incidence of thrombosis is high. Gene therapy is considered a safe strategy to increase the local expression of thrombolytic agents over an extended period of time, in parallel reducing the systemic risk of hemorrhagic complications. Ji *et al*[40] used a chitosan nano-*tPA* gene plasmid to locally transfect dog cardiomyocytes at the time of mechanical heart valve replacement. The transfected gene significantly increased the survival of animals and prevented thrombus formation on mechanical valves, without affecting systemic hemostasis. In a further study by the same group[41], the *tPA* gene plasmid was packaged in albumin nanoparticles crosslinked to ultrasonic microbubbles. Following intravenous administration, a local therapeutic ultrasound treatment of the heart after valve replacement had been performed, which resulted in increased myocardial expression of *tPA* and prevented thrombosis for 8 wk after operation.

Thrombolytic therapies

Rapid recanalization of thrombus-occluded arteries is essential to improve outcomes and reduce the mortality in acute myocardial infarction or stroke. Development of delivery systems for local thrombolysis is therefore one of the most urgent tasks in the prevention and the therapy of acute thrombotic events. Within the coagulation cascade, thrombin represents the most important target of direct anticoagulants. As an

example, hirulog, an analogue of the natural thrombin inhibitor hirudin was locally delivered to the thrombus using lipid nanoparticles containing a fibrin-binding peptide. Upon administration of the fibrin-targeting hirulog-carrying particles, significantly higher levels of antithrombin activity were achieved in the aortic tree of ApoE-deficient mice as compared with non-targeted particles[17].

The effects of another potent thrombin inhibitor, d-phenylalanyl-l-prolyl-l-arginyl-chloromethyl ketone (PPACK) were investigated the group of Myerson *et al*[42] and Palekar *et al*[43] in a mouse model of acute arterial thrombosis due to photochemical injury of the carotid artery. Perfluorocarbon nanoparticle-bound PPACK outperformed both heparin and uncomplexed PPACK in inhibiting thrombosis, and formed a local clotting barrier that remained effective even as systemic effects rapidly diminished[42]. Similarly, PPACK-liposomes administered prior to the arterial injury significantly delayed the time to arterial occlusion as compared to free PPACK. Whereas systemic anticoagulant profiles returned to control levels within 50 min, the inhibition of thrombus formation was maintained at the injury site beyond 2 h[43]. The establishment of a potent and long-acting anticoagulant surface over a newly forming clot with the use of thrombin targeted nanoparticles offers an alternative site-targeted approach to the management of acute thrombosis.

As described in detail above, intravenous infusion of tPA is characterized by several drawbacks, including low efficacy combined with a high risk of bleeding complications[35]. Therefore, several innovative strategies aiming at targeted and/or local applications of plasminogen activators have been designed. The possibility of magnetic-targeting of tPA for local thrombolysis was investigated by Ma *et al*[44] in a rat embolic model. Magnetite nanoparticles bound to tPA (tPA equivalent of 0.2 mg/kg) were administered intraarterially under guidance of an external magnet moving along the iliac artery. Magnetic tPA-nanoparticles accumulated in the thrombus-affected region and achieved an effective target thrombolysis with < 20% of a regular dose of free tPA. Another tPA delivery nanosystem comprising basic gelatin and zinc acetate was tested by Kawata *et al*[45] in a swine acute myocardial infarction model. Within this nanosystem, tPA activity was reduced *in vitro* to approximately 50% of free tPA and was fully recoverable by transthoracic ultrasound application. In comparison to treatment with free tPA (0.447 mg/kg), which recanalized the occluded coronary artery in only 1 of 10 swine, nanoparticles containing the same dose of tPA with ultrasound activation achieved recanalization in 9 of 10 swine within 30 min, suggesting that this nanosystem bears promising potential for improved intravenous thrombolysis.

In attempt to create a theranostic construct with fibrinolytic activity, McCarthy *et al*[46] synthesized a multimodal nanoagent using magnetofluorescent crosslinked dextran-coated iron oxide nanoparticles conjugated to tPA. Thrombus-targeting was achieved by nanoparticle functionalization with an activated FXIIIa-sensitive peptide. In murine models of arterial and venous thrombosis, the FXIIIa-targeted fibrinolytic nanoagent efficiently bound the margin of intravascular thrombi as detected by intravital fluorescence microscopy. The fibrinolytic activity of the nanoagent compared to free tPA was subsequently evaluated in a murine model of pulmonary embolism, showing that the FXIIIa-targeted agent lysed pulmonary emboli with similar efficacy as free tPA[46].

Targeted tPA delivery to stenotic arteries by employing universal hemodynamic phenomena was described by Korin *et al*[47]. Since occlusions in blood vessels result in local increases in shear stress, the authors designed micro-aggregates of poly-lactic-glycolic acid nanoparticles coated with tPA. Under physiologic flow conditions with shear stress values up to 70 dyn/cm^2, these micro-aggregates remained stable, but the exposure to abnormally high shear stress in the regions of vascular occlusion/stenosis resulted in their rapid break up followed by local drug release. As compared with free drug, the shear-activated tPA-coated nanoparticles rapidly dissolved the ferric chloride-induced arterial thrombi in mouse mesenteric arteries, with complete clearance of occluding thrombi within 5 min after application[47]. Moreover, upon infusion of lethally large fibrin clots, the immediate application of the shear-activated tPA-coated nanoparticles increased survival by 80%. The doses of shear-activated tPA-nanoparticles required for clot dissolution were about 100-times lower than the doses required for achieving comparable effects with free drug[47]. This strategy, utilizing a universal hemodynamic phenomenon of increased shear stress upon reduction in vessel diameter should result in a broad applicability for all occlusive vascular conditions, including *e.g.,* treatment of stenotic atherosclerotic plaques, pulmonary emboli, and ischemic stroke.

Venous thromboembolism, including deep vein thrombosis and pulmonary embolism, remains a common and potentially life-threatening disease[48]. Standard treatments aim to minimize acute morbidity and mortality by preventing the potentially fatal embolization of the initial thrombus and to reduce the long-term complications of post-thrombotic syndrome. For patients with VTE, catheter-based revascularization therapy [catheter-directed thrombolysis (CDT)] has emerged as favoured means of administration replacing systemic thrombolysis. Urokinase-type plasminogen activator (uPA) is commonly used for CDT in the clinical settings of VTE. However, similar to arterial thrombosis treatment, strict eligibility criteria are necessary to reduce the risk of bleeding complications, which limit the applicability of this therapy. To minimize the adverse effects and increase therapeutic benefits, Jin *et al*[49] produced uPA-coated, self-assembled chitosan and tripolyphosphate nanoparticles. In a

rabbit model of thrombosis, a significant improvement in the thrombolytic effect compared with free uPA was observed upon administration of uPA-carrying nanoparticles. Additionally, the study confirmed the superiority of CDT for improving clot lysis and minimizing adverse effects over drug-induced systemic thrombolysis.

CONCLUSION

The potential clinical impact of nanotechnology in terms of thrombosis prevention and management is enormous. But in spite of the promising results obtained in the vast number of bench investigations that have been published in the recent years, the thrombus-targeting imaging nanoagents and fibrinolytic nanotherapies are not yet implemented in humans. To ensure clinical safety and feasibility, the intravascular diagnostic and drug-delivery systems must be subject to a close toxicologic and pharmacologic scrutiny prior to their application in patients. Thus, substantial amount of *in vivo* studies will be necessary before the successful basic research can be translated into clinical trials. Despite multiple safety and regulatory constraints, the future progress in diagnosis and treatment of thrombosis is expected to benefit strongly from the development of novel nanotechnology-based strategies.

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P- Reviewer: Amiya E, Falconi M, Tanoglu A **S- Editor**: Gong XM
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