Prototypical Nanodevices for Targeted Delivery of Tissue Plasminogen Activator to Blood Clots

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Statement of Purpose: Currently, tissue plasminogen activator (tPA) is administered intravenously after miocardial infarction and stroke to ensure blood clot dissolution. tPA can act upon plasminogen in circulation as well as at the clot site, generating systemic plasmin and rendering the treated patient highly vulnerable to hemorrhaging. Plenty of research has been done towards the development of a more selective form of tPA. Cross-linking of tPA with the antifibrin antibody resulted in the system that was very efficient *in vitro* but failed to work *in vivo* due to low stability and uncontrollable composition of the conjugates [1]. Fusion of a monoclonal antifibrin antibody to the N-terminus of tPA [2] was also of a limited success because the attached antibody interfered with activation of tPA by fibrin.

Use of nanoparticles as protein carriers offers several advantages over the use of cross-linked or fusion proteins. Each nanoparticle can carry several protein molecules of each type, which improves targeting efficiency and renders higher local concentration of the functional enzyme in the site of action. Protein-nanoparticle conjugates often possess higher stability than free proteins and have easily controllable size and composition. Here, we study tPA and antifibrin antibody simultaneously attached to 20 nm polystyrene latex nanoparticles as potential therapeutic agent for blood clot dissolution.

Materials and Methods: Negatively charged 20 nm polystyrene latex nanoparticles with chloromethyl surface groups were purchased from Invitrogen (Eugene, Oregon). Human lyse-plasminogen (83 kDa) was obtained from Haematologic Technologies (Essex Junction, VT). The FITC-conjugated rabbit anti-human fibrinogen (AF)was purchased from Accurate Chemical Corp. (Westbury, NY). Calbiochem (La Jolla, California) supplied both the human tPA (65 kDa) and the citrate-free thrombin from human plasma (37 kDa).

tPA and fluorescently labeled AF antibody were simultaneously attached to 20 nm polystyrene latex nanoparticles via interaction of protein amine groups with the chloromethyl groups of the nanoparticles. Amount of each of the attached proteins was determined by analysis of total protein content in the conjugates (BCA assay) and fluorimetric determination of the AF antibody. Targeting of nanoparticles to fibrin clots was assayed by comparing fluorescence intensities of initial suspension of AF-tPA-nanoparticles with that of the supernatant obtained after 10 min incubation of nanoparticle suspension with the fibrin clot. Fibrin clots were prepared in microplate wells by interaction of fibrinogen and thrombin. For fibrinolysis assay, free plasminogen and suspension of antifibrin-t-PA –nanoparticles were added to a microplate well with fibrin clots. Emerging of soluble fibrin degradation products in the liquid phase collected from the well was monitored using BCA assay.

Results/Discussion: Several initial tPA/AF ratios were studied to find the optimal composition of the conjugates. The best results were received when 25 tPA and 100 AF molecules per nanoparticle were added to the initial solution. After attachment, each nanoparticle contained 20 t-PA molecules and 5 FITC-labeled AF antibody molecules. Enzymatic activity of tPA tethered to nanoparticles without effect of targeting was assessed by monitoring kinetics of reaction with S-2251 (a small molecule substrate) and consisted of ~60% of the activity of free tPA in the same conditions. In targeting assay, fluorescence of the supernatant dropped ~66% after incubation of AF-tPA nanoparticles with fibrin clots, suggesting that two-thirds of AF-tPA –nanoparticles bound to the clot.

Results of the fibrinolysis assay for AF-tPA nanoparticles as compared with free tPA are shown in Table 1. Activity of AF-tPA-nanoparticles exceeded that of free tPA at all concentrations, probably due to more effective targeting to fibrin clots. At low concentrations effect of the targeting should be more pronounced. Indeed, at 2 ng/mL tPA protein-nanoparticle conjugates are approximately four times more potent than free t-PA, while rates of fibrinolysis are almost equal at 2000 ng/mL. Notably, antifibrin-t-PA fusion protein prepared and studied by Haber et al. [2] was 2-to-3 fold less potent than free tPA in lysing clots due to impaired fibrin stimulation of the tPA portion of the recombinant molecule. This does not seem to be a problem for tPA and AF antibody simultaneously attached to the nanoparticles.

Table 1. Comparison of the initial rates of fibrin degradation for AF-tPA nanoparticles with those for free tPA at different total tPA concentrations. The conjugates contained 5 AF and 20 tPA molecules per nanoparticle.

tPA, ng/mL:	2000 ng/mL	200 ng/mL	20 ng/mL	2 ng/mL
Free tPA	$1.09\cdot10^{-3}$	$3.6\cdot10^{-4}$	$8.9\cdot10^{-5}$	$1.8\cdot10^{-5}$
Nano-AF-tPA	$1.3\cdot10^{-3}$	$7.1\cdot10^{-4}$	$1.4\cdot10^{-4}$	$6.7\cdot10^{-5}$

Conclusions: Simultaneous covalent attachment of antifibrin antibody and tPA to polystyrene latex nanoparticles results in more effective targeting of tPA to fibrin clots and higher fibrinolysis rates. This system is thus a promising therapeutic agent for post-miocardial infarction and stroke treatments.

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