Pharmacological studies of RGTA₁₁, a heparan sulfate mimetic polymer, efficient on muscle regeneration

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Abstract: RGTA is a family of chemically modified polymers that have been engineered to mimic the properties of heparan sulfates towards heparin binding growth factors. In vivo, RGTA stimulated tissue repair and protection when injected at the site of an injury. These properties have been reported in various models, suggesting a potential interest for therapeutic uses as a general tissue repair agent. We have focused our interest on RGTA₁₁, a dextran derivative that was shown to enhance, after a unique and local administration, muscle regeneration after total crushing. We first show that a single RGTA₁₁ systemic administration can be as efficient as a local injection for stimulating muscle regeneration. Using an H³-labeled RGTA₁₁ we have measured some pharmacokinetic parameters. Distribution volume was 51.81 mL, clearance was about 2 mL/min, and half-life was 94 min, giving a total elimination time of 11 h. We also demonstrate that RGTA₁₁ remains detectable in the body only after tissue injury. It was detected by autoradiography in the crushed muscle just after injury and remained at least for a week. These results provide a rational explanation for the long lasting effect of a single local or systemic injection of RGTA. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 62: 525–531, 2002

Key words: RGTA pharmacokinetic; heparan-like polymer; crushed muscle; EDL; regeneration

INTRODUCTION

Extracellular matrix (ECM) plays a key role in wound repair as a major source of growth factors, which are stored and protected by specific interactions with the glycosaminoglycan moieties of proteoglycan, particularly with heparan sulfate.¹² From these protection and storage sites, these heparan sulfate-bound growth factors, known as heparin binding growth factors (HBGFs), can be released³ and then become available to stimulate cell migration, proliferation, and differentiation. In attempts to mimic the stabilizing and protecting properties of heparan sulfates towards HBGFs, we chemically modified several polymers by controlled substitutions of reactive groups to obtain in vitro a protection of some HBGFs, including fibroblast growth factors (FGFs)⁴ and transforming growth factor beta (TGFβ).³ We focused our interest on dextran derivatives containing defined amounts of substituted carboxymethyl, benzylamide, and sulfate groups, as we found that they could by themselves stimulate tissue repair when applied at the site of the injury. These polymers were then designed as RGTA for ReGeneraTing Agents. Indeed, enhanced healing was observed in various in vivo injury models including skin,⁶ bone,⁷,⁸ colon,⁵ cornea,⁹ as well as regeneration of crushed extensor digitorum longus (EDL) and soleus muscles.¹⁰¹¹ RGTA also prevented some of the damage resulting from acute heart¹² or skeletal muscle ischemia.¹³ In addition to their HBGF protecting and stabilizing properties, RGTA were found to inhibit human leukocyte elastase,¹⁴ plasmin,¹⁵ and heparanase (Vlodavski, Meddahi, and Barritault, unpublished results). On the basis of these in vitro properties, RGTAs are believed to enhance in vivo the bioavailability of HBGFs.
Surprisingly, a single local administration was sufficient to stimulate the repair process that could only be completed after several weeks. This suggests that RGTA would act either by initially stimulating the healing process so that it would naturally proceed better and faster and then be eliminated, and/or by acting locally for a longer period. Moreover, we observed that systemic injections of RGTA performed either by intravenous or intramuscular routes were as efficient in stimulating crushed muscle regeneration as when they were administered locally. It was therefore tempting to hypothesize that RGTA would stick and remain in the injured tissue, through interactions at the multiple heparin binding sites identified on most matrix proteins. These sites should have become available for RGTA only after the natural endogenous and bound heparan sulfate had been degraded following the tissue injury. Using tritiated RGTA, we now provide evidence to support the hypothesis of RGTA remaining at the site of injury, and we also present some initial pharmacokinetic data.

MATERIALS AND METHODS

Synthesis and radiolabeling of RGTA

The water soluble dextran-derivative was prepared from T40 dextran (Pharmacia) as previously described. Briefly, dextran units were substituted with carboxymethyl (CM) groups followed by adding hydrophobic benzylamide groups (CMB) on CM residues and then sulfonate groups (CMBS). The chemical composition was determined by acidimetric titration for the residual unsubstituted carboxylic functions and element analysis of nitrogen (which represented a measurement of benzylamide content) and sulfur, for sulfate content. The final compound, named RGTA, was composed of various amounts of CM, CMB, and CMBS units. For RGTA11, used in these experiments, the percentage of hydroxyl groups bearing substitutions was 110% for CM, 2.5% for B, and 36.5% for BS groups. It was selected for its low anticomplementary activity, its very low anticoagulant activity (4 IU/mg vs. heparin as control), and its ability to mimic heparin or heparan sulfate proteoglycans in their in vitro interactions with FGFs.

For pharmacokinetic experiments RGTA11 was radiolabeled by moderate proton bombing (Sib Tech Inc, NJ). The H3-RGTA11 obtained had a specific activity of 20 mCi/mg. This compound was kept at ~20°C, and its biological activity was tested as previously described by Tardieu et al. The major advantage of this technology is that it provides labeled molecules identical to the nonlabeled molecules, as the bombing is moderate and preserves all physical and biological properties.

Crushing EDL muscle model

All experiments described below were performed in accordance with the rules of the European Community for animal experimentation [(rule N° 86/609/CEE]. Male Wistar rats (Wi/Wi, Ico, IFFACREDO, France), weighing 250 g, were anesthetized by intraperitoneal injection of 100 µL/100 g weight of pentobarbital sodium solution (9 g/mL; Sanofi santé animale, France). The EDL crush was performed as previously described. Briefly, after sectioning the motor nerve, EDL muscle was released from the anterior portion of the leg and injured mechanically by application of a constant pressure on all the length of the muscle with Pén forceps. The pressure was maintained for approximately 15 s. Tendons were maintained in place but vascularization and innervation were suppressed. The muscle was then replaced and only the cutaneous level was sutured using Prolène® 3/0 (Ethicon, France), to avoid a lodge syndrome consequence of local inflammation.

Histological studies of EDL regeneration after intravenous RGTA11 treatment

Immediately after EDL crushing, 10 Wistar rats (Wi/Wi, Ico, IFFACREDO, France) weighing 280–300 g were treated by intravenous injection in the penis vein of 200 µL of 12.5 mg/mL RGTA11, and 10 other Wistar rats received saline solution as a control. This site was found easier to control when the entire sample was injected.

Eight days after the injury, the EDL muscle was removed, sectioned in its midpart and rapidly frozen in liquid isopentane cooled by liquid nitrogen at −150°C. Sample sections of 7 µm thick were made using a cryostat (Leica, France). Serial sections performed in the midregions were stained with Gomori’s trichrome and examined using a light microscopy.

H3-RGTA11 pharmacokinetic study

Twelve Wistar rats (Wi/Wi, Ico, IFFACREDO, France) weighing 280–300 g were used in each study. Animals were anaesthetized by intraperitoneal injection of pentobarbital sodium solution (100 µL/100 g weight; Sanofi santé animale, France).

To allow blood sample removal, the carotid artery was catheterized (Jelco Winged; Johnson & Johnson) and 500 µL of blood was collected in a heparinized Eppendorf tube, centrifuged, and 50 µL of plasma was counted in a beta scintillation counter (EG&G, Wallac, France) after addition of 5 mL of Hionic Fluor (Packard, France). This sample corresponded to the zero time of the pharmacokinetic studies.

H3-RGTA11 (1.5 × 106 cpm) was injected through the penis intravenously (i.v.) after realization or not of the EDL crushing lesion. Blood samples were drawn by carotid catheter at 1, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, and 360 min after the i.v. administration and then centrifuged. The plasma radioactivity was measured as described above. During all experiments, blood volume was maintained by injection of the same volume of saline solution after each removal.

Pharmacokinetic analysis

Equation (1) describes the time course of the H3-RGTA11 concentration in the blood according to a two compartments
open model. The parameters were determined using the nonlinear least-squares program Micropharm.\textsuperscript{18} The data were ascribed to the equation

\[ C_t = A e^{-\alpha t} + B e^{-\beta t} \]  

(1)

A and B were constants. The elimination half-time period, \( t_{1/2} \beta \) (\( \beta \) phase), corresponds to the time taken for the concentration of drug in plasma to decline to half of its original value and was calculated from the parameter \( \beta \) by the equation

\[ t_{1/2} \beta = \frac{\ln 2}{\beta} \]  

(2)

From this measure, a given substance is completely eliminated after a time of \( 7 t_{1/2} \beta \).

The clearance (Cl) is the elimination of the drug in mL/min and can be expressed by Equation (3):

\[ Cl = \frac{AUC}{D} \]  

(3)

where AUC stands for the Area Under the Curve and is calculated using the trapezoidal rule by the Micropharm program,\textsuperscript{18} and D is the administered dose of H\textsuperscript{3}-RGTA\textsubscript{11}.

The apparent distribution volume (VD) is the ratio of the amount of drug in a whole organism and its blood or plasma concentration measured at the same time.

Results were analyzed by ANOVA statistical test (Statview).

H\textsuperscript{3}-RGTA\textsubscript{11} localization in crushed EDL muscle

Eight Wistar rats were used for this study. After left EDL crushing, \( 3 \times 10^6 \) cpm of H\textsuperscript{3}-RGTA\textsubscript{11} diluted in 200 mL of saline solution was injected intravenously in the penis vein. This injection was followed by flushing the syringe with 200 mL of saline solution. Left crushed muscle and right control muscle were removed at various times between day 1 and 9 after injury, sectioned in their midpart, and rapidly frozen as described above.

Serial sample sections performed in the midregion were realized and dried, and autoradiographies were performed for each section during 72 h with a real-time radioimager (Micro\textsuperscript{2}imager; Biospaces mesures, France). Quantification of the radiography was analyzed by the Biospace software.

RESULTS

Histological studies of EDL regeneration after RGTA\textsubscript{11} treatment

Figure 1(3,4) shows regenerated EDL muscles treated with intravenous injection of RGTA\textsubscript{11} after 8 days. The treatment with RGTA enhanced the number and the diameter of the regenerated muscle elements. At this time, the myotubes differentiated in myofibers (MF). Some centralized myonuclei (CN) persisted, but many of them were already at the border (LN). The treatment also increased the stage of differentiation of the perimysium (PY). This connective tissue was well formed and separated the bundles of the muscular fibers. Blood vessels (V), arteries, and veins, were already well formed in the presence of differentiated endothelial cells (E). In non-RGTA treated EDL muscles [Fig. 1(1,2)], regeneration was delayed. Histological sections show less and smaller newly formed myotubes (MY) separated by interstitial tissue (I) containing many mononucleated cells (MC). These poorly differentiated myotubes have central nuclei (CN). Some myotubes are clustered before their secondary fusion to form larger myofibers (CL).

DISCUSSION

Our initial observations pointed out that a single and local injection of RGTA\textsubscript{11} in a crushed muscle stimulated muscle regeneration.\textsuperscript{10} RGTA\textsubscript{11} was active in a bell shape dose-dependant manner with optimal dose obtained by injecting 20 \( \mu \)L of a solution at 500 \( \mu \)g/mL in the crushed muscle. We now present similar effects obtained by a single systemic injection at an optimal dose of 2 mg/kg and data supporting the hypothesis that RGTA\textsubscript{11} binds locally only at the sites where the tissue had been injured. The use of a sys-
Figure 1. Histological studies of the post-traumatic regeneration of control or intravenously RGTA treated EDL muscles of rat. Sections of medial parts of frozen tissues were stained with Gomori’s trichrom. (1,2) Regenerated non -RGTA_11 treated EDL muscles 8 days after crush. The micrographs show smaller newly formed myotubes (MY) separated by interstitial tissue (I) containing many mononucleated cells (MC). These poorly differentiated myotubes have central nuclei (CN). Some myotubes are clustered before their secondary fusion to form larger myofibers (CL). (3,4) Eight day regenerated EDL muscles treated with intravenous injection of RGTA_11. The treatment with RGTA_11 enhanced the number and the diameter of the regenerated muscle elements. At this time, the myotubes are differentiating in myofibers (MF). Some centralized myonuclei (CN) persist, but many of them are lateralized (LN). The treatment also increased the differentiation of the perimysium (PY). In this connective tissue separating the bundles of the muscular fibers the blood vessels (V), arteries, and veins are formed with the presence of well- differentiated endothelial cells (E). (1,3) Original magnification ×250. (2,4) Original magnification ×500.
temic injection and the evidence of a specific targeting of RGTA 11 to the damaged tissue widen the interest for clinical general uses.

A muscle is a well-delimited tissue and local optimal dose injection of RGTA 11 in a crushed muscle induced after a week a 10-fold increase in the number of muscle fibers and bundles formed versus the controls. Pharmacokinetic studies indicate that H3-RGTA11 used at trace amount can be measured in the plasma as a function of time and its elimination exhibited a biexponential decrease curve with two phases (Fig. 2). The first phase is an initial fast decrease and includes the distribution of RGTA from the plasma in a second compartment (tissue). The second phase, almost linear, provides the value of the elimination rate of H3-RGTA11 at the steady state. Study of the apparent VD confirmed the diffusion of H3-RGTA11 in a second compartment (Table I) and a quick elimination (t1/2β = 94 min). Complete plasmonic elimination of H3-RGTA11 was obtained in 11 h. These pharmacological results were similar to those obtained with anionic dextran such as sulfate dextran or heparin. Indeed, sulfated or not, dextran is generally analyzed by a bicompartment model after i.v. administration. In our experiments, H3-RGTA11 was injected at tracer doses and it was not possible to obtain specific activities with tritium that permitted an injection of effective pharmacological doses. The proportion of labeled material stuck in the injured muscle was still too small to make a difference detectable at the plasma level when compared to healthy controls. There was also no significant difference in the amount of radioactivity measured in various other organs (heart, brain, kidney, liver) between noninjured and EDL crushed animals (data not shown). It was only after autoradiographies of the histological sections of the damaged EDL muscle that H3-RGTA11 was clearly detected and absent in the contralateral intact EDL. H3-RGTA11 binds only at the site of the injury and was detected very early (Fig. 3(A1)). Nine days later H3-RGTA11 was still detected (Fig. 3(A4)). The selective fixation of RGTA11 at the sites of injury and not elsewhere provides a good rational to explain the long lasting effects of RGTA11 and the bell shaped dose response curve that we have always observed in our various in vivo tissue repair models. We can indeed speculate that RGTA11 will bind to the multiple heparin binding sites of the matrix proteins (such as those of laminin, fibronectin, and various collagens, etc.), which one expects to be accessible after heparanase degradation of the heparan sulfates normally present in steady state situations. It is likely that H3-RGTA11 can only stick at these sites. Similar distributions were obtained after injection of labeled heparin in normal and damaged tissues.

Once RGTA11 is bound at these sites, it remains and protects the matrix protein from further degradation by steric blockage and by more specific proteases inhibitions such as plasmin and elastase. Furthermore, it allows the binding of HBGFs, which in turn can stimulate tissue repair. We have indeed observed a better protection of muscle fibers in acute ischemia and of the basal membrane in RGTA11-treated crushed muscles (Aamiri and Gautron, in preparation). We can therefore suggest that one possible mechanism of action for RGTA11 would solely be to replace the heparan-sulfate and protect the basal membrane from further degradation. HBGFs would naturally be protected and available to be presented to their high affinity cellular receptors. Furthermore, RGTA11 in excess would not remain but may by competition displace and carry out HBGFs from their local place into the blood circulation. Bell shaped dose-dependant RGTA regeneration may be therefore simply explained by this wash-out hypothesis. However, one has to consider these studies as preliminary and that these hypotheses would need more experimental support.

RGTA11s represent a potential new class of therapeutic agents, and it is through a better understanding of their modes of action and their pharmacological properties that this family of agents will eventually be developed for human use.

### TABLE I

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<th>Pharmacokinetics Parameters of RGTA11</th>
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<td>IV Control Muscle</td>
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References


Figure 3. EDL muscle autoradiographies after H3-RGTA11 intravenous injection. [A(1,2,3,4)] Crushed EDL. [A(5,6,7,8)] Intact EDL. (B) Quantification of H3-RGTA11 fixation in EDL section samples after muscle lesion (black bar) or intact muscle (white bar) in function of days after injury. There was no significant difference (i.e., the measure of radioactivity in the whole muscle slice was not significantly different) between days 1, 4, 6, and 9.

