RGD-peptides modifying dexamethasone: to enhance the anti-inflammatory efficacy and limit the risk of osteoporosis
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Dexamethasone (Dex) is one of the most effective anti-inflammatory glucocorticoids, while the side effect, osteoporosis seriously limits its clinical use. Cell adhesion is involved in the onset of inflammation and osteoporosis, and RGD-peptides are well known as anti-adhesion peptides. To enhance the anti-inflammatory efficacy and limit the osteoporotic risk of Dex three novel conjugates of RGDV, RGDS and RGDF covalently modified Dex are presented here. For the xylene-induced ear edema model the ear edema of the mice treated with the conjugates was significantly lower than that of the mice treated with Dex. Receiving 15 day therapy the total volumetric bone mineral density, the peripheral quantitative CT and the femur weight of the mice treated with the conjugates were significantly higher than those of the mice treated with Dex. Therefore covalently modifying Dex with RGDV, RGDS and RGDF not only increased the anti-inflammatory activity but also decreased the osteoporotic risk of Dex. In addition, the enhanced anti-inflammatory activity was correlated with the down-regulated DNA expression of the conjugates.

Introduction

Dexamethasone (Dex) represents the most effective anti-inflammatory glucocorticoid for treating several chronic and acute inflammatory conditions,1–3 peritoneal adhesion, cardio-pulmonary bypass and acute infection,4–6 and rheumatoid arthritis.7–11 However, the clinical efficacy of Dex is limited by a series of side effects.12 Of side effects osteoporosis is capable of weakening trabecular bone and increasing the fracture risk of the spine, hip and rib.13–15 To eliminate the osteoporotic risk Dex has been converted to various preparations, such as encapsulations and liposomes,16 hydrophilic gold nanoparticles,17 copolymer and N-(2-hydroxypropyl) methacrylamide conjugates,18 and was conjugated with β-cyclodextrins,19 and polyethylene glycol.20 Nevertheless the risk of osteoporosis remains the most serious drawback of Dex therapy.

RGD-tetrapeptides, well known anti-adhesion molecules, are a motif of integrins recognizing collagen, fibronectin, vitronectin, laminin, immunoglobulin superfamily and plasma proteins. The motif like function of RGD-tetrapeptides to integrins has been widely used in drug design. The use of the anti-adhesion property of RGD-tetrapeptides to integrins resulted in the design of biomaterials such as the amphiphilic block copolymer,21 hydroxyapatite biomaterials,22 collagen tubes,23 conjugates that promote cell adhesion and cell spreading,24 mussel adhesive proteins,25 as well as resulting in anti-thrombotic agent design.26–29

Cell adhesion is involved in the onset of both inflammation and osteoporosis. In order to enhance the anti-inflammatory activity and limit the osteoporotic risk, the present paper reports the covalent modification of Dex with the anti-adhesion peptides RGDV, RGDS and RGDF to form three novel conjugates, the characterization of their nanostructures, and the evaluation of their anti-inflammatory activities, estimates their osteoporotic risk and explores the possible mechanism.

Results and discussion

Synthesis of the conjugates

By using the 9-step procedure depicted in Scheme 1 RGD-peptide modified Dex, 4a (RGDV-Dex), 4b (RGDS-Dex) and 4c (RGDF-Dex) were prepared in acceptable yields. The modified
hydroxyl of Dex was identified in the ROESY 2D NMR spectra of 4a-c, the cross-peaks were marked with blue rings and are shown in Fig. S13–S15,† which consistently shows a cross peak from –CO–CH₂–O (4.20 ppm) and –O–CO–CH₂–CH₂–CO– (2.16 ppm). Therefore among the 11-hydroxy, 17-hydroxy and 17-[2-hydroxycetyl] of Dex only the latter was modified by RGD-peptides. The details and the characterisation data are given in the ESI.†

**FT-MS spectral evidence in water showing the conjugates form trimers**

The mass spectra of RGDV-Dex, RGDF-Dex and RGDS-Dex were obtained on a Solarix FT-ICR mass spectrometer (Bruker Daltonics) with ESI ion source and a superconductive magnet of 9.4 T. Fig. 1 shows that the FT-MS spectrum of a solution of RGDV-Dex or RGDS-Dex or RGDF-Dex in ultrapure water gives the ion peak of the trimer. Fig. 1 also shows that the spectrum gives the ion peaks of the dimer and the monomer, the qCID spectra demonstrate that both the dimer and the monomer are the fragmentation products of the trimer. Therefore the trimer is the existing form of RGDV-Dex, RGDF-Dex and RGDS-Dex in ultrapure water.

**ROESY 2D NMR clarifies the manner of trimerization**

To reveal the manner of the trimer formation the ROESY 2D NMR spectra of RGDV-Dex, RGDF-Dex and RGDS-Dex were measured at 800 MHz in deuterated DMSO. Fig. 2a shows the NOESY 2D NMR spectra of the 3 conjugates, and each gives one interesting cross-peak only, which are labeled with blue circles, and mirrors the interaction between the H of CO–CH₂–CH₂–CO of one molecule with the C-terminal carboxyl H of RGD-tetrapeptide moiety of another molecule. This means that the interaction of CO–CH₂–CH₂–CO and the C-terminal amino acid of the peptide moiety of 3 molecules of RGDV-Dex or RGDF-Dex or RGDS-Dex approach each other and form the trimers. The related NMR spectra are also given in the ESI.† The 3D-features of the trimers were constructed with the energy optimized 3 monomers approaching each other in the manner defined by the NOESY 2D NMR spectra. Fig. 2b shows that the trimers look like umbrellas.

**TEM image reveal the conjugates forming nanoparticles**

The TEM images of 1 nM aqueous conjugates were measured. Fig. 3 shows that in ultrapure water RGDV-Dex, RGDF-Dex and RGDS-Dex form nanoparticles of 18.2–98.2 nm, 54.4–145.7 nm and 20.2–71.4 nm in diameter, respectively. Having diameters less than 150 nm and in the most case less than 100 nm means that the nano-particles can not be phagocytized by macrophages and should be safely delivered by blood circulation.

**Relationship between trimers and nanoparticles**

The Mesocite module of the Materials Studio software was used to show the process of the trimers forming nanoparticles and to predict the number of the trimers involved in a definite nanoparticle, for which RGDV-Dex, RGDF-Dex and RGDS-Dex were built and optimized simply in the Visualizer window. The “beads” were constructed from atomistic simulations and placed at the center-of-mass of groups of atoms corresponding to particular parts of the molecules of RGDV-Dex, RGDF-Dex and RGDS-Dex. Fig. 3a–c show that a nanoparticle of 5.67 nm in diameter of RGDV-Dex contains 110 trimers, a nanoparticle of 5.72 nm in diameter of RGDF-Dex contains 111 trimers, and a nanoparticle of 5.78 nm in diameter of RGDS-Dex contains 104 trimers. Unexpectedly, the 3 simulated nanoparticles occur as the smallest nanoparticles in Fig. 3a–c. Therefore Mesocite simulation can help us to understand the relationship between the trimer and the nanoparticles of RGDV-Dex, RGDF-Dex and RGDS-Dex.

**RGD-tetrapeptide covalent modification enhances the anti-inflammatory activity**

To examine the effect of RGD-tetrapeptide covalent modification on the clinical use of Dex in treating inflammatory diseases the anti-inflammatory activities of Dex and the conjugates were evaluated with xylene-induced ear edema assay. In
brief, the mice were orally administered with 0.5% CMC-Na (blank control), or a suspension of 25.5 μmol kg⁻¹ of Dex in
0.5% CMC-Na, or a suspension of 25.5 μmol kg⁻¹ of RGDV-Dex in 0.5% CMC-Na, or a suspension of 25.5 μmol kg⁻¹ of

Fig. 1 FT-MS spectra of solutions of RGDV-Dex, RGDF-Dex and RGDS-Dex in ultrapure water: spectrum of RGDF-Dex gives an ion peak of the trimer plus 2Na and H at [1012.41154]+3, an ion peak of the dimer plus H at [1029.47134]+2
and an ion peak of the monomer plus H at [990.41826]+1; spectrum of RGDS-Dex gives an ion peak of the trimer plus 3Na at [930.39186]+3, an ion peak of the dimer plus Na and K at [941.37277]+2 and an ion peak of the monomer plus H at [908.41004]+1; spectrum of RGDV-Dex gives an ion peak of the trimer plus 3K at [957.07586]+3, an ion peak of the dimer plus Na and H at [931.43343]+2 and an ion peak of the monomer plus H at [919.51148]+1.

Fig. 2 (a) ROESY 2D NMR spectra: the cross-peaks labeled with blue circles define the intermolecular interaction of RGDV-Dex, RGDF-Dex and RGDS-Dex; (b) the 3D-features of the trimers of RGDV-Dex, RGDF-Dex and RGDS-Dex at minimal energy look like umbrellas.

Fig. 3 TEM images of 1 nM solution of the conjugates in ultrapure water: (a) TEM image of RGDV-Dex; (b) TEM image of RGDS-Dex; (c) TEM image of RGDF-Dex. Calculated nanoparticles of the conjugates: (a’) calculated nanoparticle of 5.67 nm in diameter of RGDV-Dex; (b’) calculated nanoparticle of 5.72 nm in diameter of RGDS-Dex; (c’) calculated nanoparticle of 5.78 nm in diameter of RGDF-Dex.
RGDF-Dex in 0.5% CMC-Na, or suspensions of 0.25, 2.5 and 25.5 μmol kg\(^{-1}\) of RGDS-Dex in 0.5% CMC-Na, the ear edema of the mice were measured and are shown in Fig. 4B. The data indicate that 25.5 μmol kg\(^{-1}\) Dex effectively exhibits xylene-induced ear edema, but the efficacy is significantly lower than that of 25.5 μmol kg\(^{-1}\) RGDV-Dex, RGDS-Dex and RGDF-Dex, and equals that of 2.55 μmol kg\(^{-1}\) RGDS-Dex. Therefore the anti-inflammatory activity of Dex is increased by 10 fold due to the covalent modification. Fig. 4B also indicates that the plasma TNF-α and IL-8 of the inflammatory mice orally treated with 25.5 μmol kg\(^{-1}\) Dex effectively exhibits xylene-induced ear edema and significantly lower than those of NS treated inflammatory mice and sham mice, but is significantly higher than those of 25.5 μmol kg\(^{-1}\) RGDS-Dex, RGDV-Dex and RGDF-Dex treated inflammatory mice. Thus it is hypothesized that via decreasing plasma TNF-α and IL-8 RGD-tetrapeptide covalent modification enhances the anti-inflammatory activity.

Fig. 4 (A) DNA concentrations of the A549 cells treated with 1 μM Dex, RGDV-Dex, RGDS-Dex and RGD-Dex, \(n=4\); (B) anti-inflammatory activities of Dex, RGDV-Dex, RGDS-Dex and RGD-Dex, \(n=12\); (C) plasma TNF-α of the inflammatory mice orally treated with CMC-Na, Dex, RGDV-Dex, RGDS-Dex and RGD-Dex, \(n=12\); (D) plasma IL-8 of the inflammatory mice orally treated with CMC-Na, Dex, RGDV-Dex, RGDS-Dex and RGD-Dex, \(n=12\); (E) ESI-MS spectrum of cytoplasm of A549 cells treated with NS, \(n=4\); (F) ESI-MS spectrum of cytoplasm of A549 cells treated with 1 μM RGDV-Dex, \(n=4\); (G) ESI-MS spectrum of cytoplasm of A549 cells treated with 1 μM RGDS-Dex, \(n=4\); (H) ESI-MS spectrum of cytoplasm of A549 cells treated with 1 μM RGDS-Dex, \(n=4\).

Down-regulating DNA replication and enhancing in vivo anti-inflammatory activity

A549 cells have been widely used to respond to the cross talk between inflammation and DNA replication,\(^{30–32}\) and some anti-inflammatory agents were reported to be able to counteract both the pro-inflammatory effect and DNA replication.\(^{33}\)

To correlate the enhanced in vivo anti-inflammatory activity with DNA replication the quantities of the DNA of treated A549 cells were measured. Fig. 4A indicates that the DNA quantity of A549 cells treated with 1 μM Dex is significantly lower than that of A549 cells treated with NS (\(p<0.05\)), but is significantly higher than that of A549 cells treated with 1 μM RGDV-Dex, RGDS-Dex and RGD-Dex (\(p<0.01\)). Thus down-regulating DNA replication may be one of the mechanisms of RGD-tetrapeptide modification to enhance the in vivo anti-inflammatory activity. This was further ensured by recording ESI-MS spectra of the cytosome of treated A549 cells. Fig. 4E–H show that the ESI-MS spectra of the cytosome of A549 cells treated with RGDV-Dex, RGDS-Dex and RGD-Dex, but not with NS, give the ion peak of Dex, i.e. the ESI-MS spectrum of the cytosome of NS treated A549 cells does not give any Dex related ion peak (Fig. 4E), while the ESI-MS spectra of the cytosome of A549 cells treated with 1 μM RGDV-Dex, RGDS-Dex and RGD-Dex consistently give the ion peak at ~807.42, the mass of a dimer of Dex plus Na (Fig. 4F–H). This suggests that the trimers of RGDV-Dex, RGDS-Dex and RGD-Dex enter the cells, release the dimers of Dex and down-regulate the replication of A549 cell DNA.
The effect of RGD-tetrapeptide covalent modification on the osteoporotic risk of Dex therapy was examined with a mouse model. In brief, BALB/C mice (male, 14 weeks in age) were orally administered with 0.5% CMC-Na or a suspension of 1.43 μmol kg⁻¹ per day of Dex in 0.5% CMC-Na or a suspension of 1.43 μmol kg⁻¹ per day of the conjugates in 0.5% CMC-Na for 15 consecutive days to record the total volumetric bone mineral density (vBMD) and the peripheral quantitative CT (pQCT) images of the femurs. Fig. 5 indicates that the femurs of the mice receiving 0.5% CMC-Na, and 1.43 μmol kg⁻¹ per day of RGDV-Dex or RGDS-Dex or RGDF-Dex have close total vBMD values and similar images, suggesting during 15 day treatments they induce no femur loss, while the total vBMD value and image of the femurs of the mice receiving 1.43 μmol kg⁻¹ per day of Dex are lower than that of the mice receiving 0.5% CMC-Na, suggesting during 15 day treatments Dex induces femur loss. Therefore RGD-tetrapeptide covalent modification effectively decreases the osteoporotic risk of Dex therapy.

Experimental

The detailed methodologies and data for all experiments are given as ESI†

Conclusions

The covalent modification of Dex with RGDV, RGDS and RGDF can effectively increase the anti-inflammatory activity and lower the osteoporotic risk of Dex therapy, and therefore is a general strategy to improve the clinical anti-inflammatory therapy of glucocorticoids. The enhancement of the anti-inflammatory activity may be attributed to the abilities of RGDV-Dex, RGDS-Dex and RGDF-Dex to cross membrane and down-regulate DNA replication. To clarify the integrin responsible for RGDV-Dex, RGDS-Dex and RGDF-Dex having a higher anti-inflammatory activity and less side effects is of pharmacological importance, is one of our interests and should be deeply investigated.

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Notes and references


