Integrin-targeted nanocomplexes for tumour specific delivery and therapy by systemic administration

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Abstract

Nanoparticle formulations offer opportunities for tumour delivery of therapeutic reagents. The Receptor-Targeted Nanocomplex (RTN) formulation consists of a PEGylated, endosomally-cleavable lipid and an RGD integrin-targeting, endosomally-cleavable peptide. Nanocomplexes self-assemble on mixing with plasmid DNA to produce nanoparticles of about 100 nm. The environmentally-sensitive linkers promote intracellular disassembly and release of the DNA. RTNs carrying luciferase genes were administered intravenously to mice carrying subcutaneous neuroblastoma tumours. Luciferase expression was much higher in tumours than in liver, spleen and lungs while plasmid biodistribution studies supported the expression data. Transfection in tumours was enhanced two-fold by integrin-targeting peptides compared to non-targeted nanocomplexes. RTNs containing the interleukin-2 (IL-2) and IL-12 genes were administered intravenously with seven doses at 48 h intervals and tumour growth monitored. Tumours from treated animals were approximately 75% smaller on day 11 compared with RTNs containing control plasmids with one third of treated mice surviving long-term. Extensive leukocyte infiltration, decreased vascularization and increased necrotic areas were observed in the tumours from IL2/IL12 treated animals. Splenocytes from re-challenged mice displayed enhanced IL-2 production following Neuro-2A co-culture, which, combined with infiltration studies, suggested a cytotoxic T cell-mediated tumour-rejection process. The integrin-targeted RTN formulation may have broader applications in the further development of cancer therapeutics.

1. Introduction

Nanoparticles based on formulations of DNA or RNA with poly-cationic polymers or liposomes have potential for the delivery of nucleic acid therapeutics to tumours by systemic administration. Such formulations would be particularly useful for targeting inaccessible or multi-focal tumours, including metastases. Poly-cationic formulations that avoid the first-pass organs, such as liver, lung, and spleen, employ shielding moieties such as polyethylene glycol (PEG) that enable longer term survival of particles in the circulation, enhancing the opportunity to target tumours [1]. PEG modification, however, frequently compromises transfection efficiency and so recent strategies have focused on formulations in which the PEG shielding moiety is removed by cleavage of chemical linkers inside the cell environment [2].

The Receptor-Targeted Nanocomplexes (RTNs) are formulated from a mixture of a liposome (ME42/DOPE), an integrin-targeting peptide (ME27) and plasmid DNA [3,4]. The RTN is a multifunctional nanoparticle delivery system, that has stealth properties from “short PEG” (triethylene glycol moieties), integrin receptor-targeting properties, and cleavable linkers to promote particle disassembly in response to the intracellular environment, thereby increasing transfection efficiencies [3]. The objective of this study was to further assess tumour targeting and the therapeutic potential of tumour gene therapy with RTN formulations. Here we evaluated plasmids encoding for human interleukin-2 (IL-2) and murine, single-chain IL-12, which were previously demonstrated to be therapeutic for murine neuroblastoma tumours in cell-mediated delivery strategies [5,6]. Interleukin-12 is a potent activator of natural killer (NK) cells and T lymphocytes and promotes the development of CD4+ Th1 inducing anti-tumour immunity [7] through the actions of IFN-γ but it can also synergize with IL-2 to generate lymphokine-activated killer (LAK) cells against tumour targets [8]. Furthermore, IL-2 activates and enhances the cytolytic
activity of NK and T cells [9,10] with significant anti-tumour affects [11–13]. We have shown previously that cell-mediated strategies involving intratumour injection into syngeneic tumours in A/J mice of Neuro-2A neuroblastoma cells [6] and fibroblasts [5] transfected with genes for IL-2 and IL-12, mediated immune effects against established tumours leading to tumour eradication and retardation. The properties of RTNs carrying IL-2 and IL-12 genes (RTN:II2/II12) potentially allow for direct delivery to tumours by i.v. administration as a pharmaceutical preparation.

2. Materials and methods

2.1. Cell culture

The murine neuroblastoma cell line (Neuro-2A) was obtained from ATCC, (Ted-dington, UK), and was maintained as described previously [6].

2.2. Peptides, lipids, plasmids

Cleavable peptide ME27, (K)4RVRGACKGDCLG, described previously [3], was custom-synthesized by ImmuN Kontakt (Abingdon, UK). PEyGalated cleavable lipid, ME42 (DOSE3P) was synthesized and formulated as a liposome with DOPE at a 1:1 ratio as described previously [4]. The plasmids pCILuc [3], pC-HIL-2 plasmid [6] and pC-mIL-12 were described previously [5,14]. RTNs were made as described previously [3].

2.3. In vivo transfections

In vivo transfections and analysis of tumour and organ luciferase expression were performed as described previously [3]. For cytokine gene immunotherapy experiments injections were started when tumours were approximately 2 × 2 mm and tumour progression was monitored as previously [5,6].

2.4. Quantitative real time polymerase chain reaction (qPCR)

DNA was isolated from tissues using the DNeasy tissue kit from Qiagen (Crawley, UK). The sequences used were: forward primer 5’-GGCCGGTTATTTATCGGACT-3’; reverse primer 5’-CCATCTTTGACAAATTCAGTT-3’; probe: 5’-FAM-CTGGCCCGCGAACGAC-CACTTATAAT-TAMRA-3’. The qPCR assay conditions were: stage 1, 95 °C for 2 min; stage 2, 95 °C for 10 min; stage 3, 95 °C for 15 s, then 60 °C for 1 min; repeated 40 times. The plasmid pCILuc was used as a standard. qPCR was performed in triplicate in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

2.5. Measurement of cytokine expression in tumour explants

Tumours were finely minced and cultured in 1 ml of DMEM with Glutamax-1 (Invitrogen, Paisley, UK) supplemented with 10% FCS (Sigma, Poole, UK), 1% non-essential amino acids, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Paisley, UK) in 6-well plates. Medium was removed every 24 h over three days and cytokine expression levels were determined by DuoSet ELISA Development System (R&D Systems, Abingdon, UK).

2.6. Leukocyte infiltration in tumours

Tumours were washed through cell strainers and erythrocytes lysed on ice for 5 min with 0.83% ammonium chloride. Cells were incubated with 1:15 dilution of PerCP-anti-mouse CD45 (Pharmingen, San Diego, CA, USA) for 30 min at 4 °C, washed and fixed in 1% PFA in PBS then analyzed by flow cytometry.

2.7. Histological analysis

Tumours or livers were excised, and stained with haemotoxylin and eosin, as described previously [5]. The Evan’s Blue Assay to assess tumour leakiness was performed as described previously [15].

2.8. Enzyme-linked immunosorbent spot assays (ELISpot)

The procedure was performed with splenocytes as described previously [5].

2.9. Statistical analysis

All experiments were carried out at least three times and the results are shown as means ± standard deviation. The statistical significance of results was assessed using the Student t-test or Mann–Whitney U-test for non-parametric data, and results were taken to be significant when p < 0.05.

3. Results

3.1. Analysis of formulations

RTNs were formulated in a weight ratio of 2:4:1 (lipid:peptide:DNA) as optimized previously in Neuro-2A cells [3]. RTN:pCILuc formulations (ME42/DOPE:ME27:pCILuc) displayed a modal particle size of 101 nm (with 90% of the particles smaller than 179 nm) analyzed by Nanoparticle Tracking Analysis (NTA) (Supplementary Methods and Fig.1s).

3.2. In vivo dosage and transfection of tumour cells with RTNs

RTN:pCILuc in 100 µl 5% glucose was injected into A/J mice bearing subcutaneous (s.c.) tumours. Luciferase expression was measured in tumour extracts 24 h later (Fig. 1A). The 50 µg dose was significantly better than 20 µg (p < 0.05), while there was no further improvement at 100 µg. Ten mice were then injected i.v. with 50 µg of pCILuc in RTN. After 24 h excised tumours were sliced into equal sized quadrants and processed for luciferase expression. Two of the mice (Mice 1 and 7) had luciferase expression in one quadrant, six in three quadrants (Mice 2, 4, 5, 6, 8 and 10) and two in all four quadrants (Mice 3 and 9) (Fig. 1B) while expression levels within quadrants from the same tumours varied up to ten-fold in nine mice, but by almost 100-fold in mouse 5. The Evan’s Blue assay demonstrated a “leaky” vasculature within the tumour (Fig. 2S).
3.3. Comparison of tumour transfection efficiencies of naked DNA and transfection complexes

Transfection complexes (50 μg pCILuc) were administered to assess the transfection efficiency at 24 h of ternary RTN formulations compared to binary formulations of peptide:pCILuc and lipid: pCILuc and naked plasmid. Naked plasmid pCILuc and ME27:pCILuc peptide complexes were more than a hundred-fold lower than RTN (ME42/DOPE:ME27:pCILuc) while ME42/DOPE-pCILuc lipid complexes (162,600 RLU/tumour) were more than 25-fold lower (p < 0.01) (Fig. 2). To evaluate integrin-mediated tumour targeting, the ME27 peptide of the RTN was replaced with the non-targeting K16 peptide and compared in transfecting tumours similar in size (p > 0.05). The integrin-targeting RTN (ME42/DOPE-ME27:pCILuc) transfected tumours 2.4 times more efficiently (p < 0.05) than the non-targeting RTN (Fig. 2).

3.4. Biodistribution of administered RTN

Mice were administered i.v. with RTN:pCILuc (50 μg of DNA) and sacrificed 24 h later for biodistribution studies. qPCR analysis of plasmid copy number, standardized to genomic DNA content, was compared to the distribution of luciferase expression, shown as total expression per tumour or organ (Fig. 3). Plasmid was primarily
found in tumour at levels 2.5-fold higher than in lung and spleen and 4-fold higher than in liver (p < 0.001). However, luciferase enzyme activity (RLU/organ) in lung was only 0.25% of that in tumour, while liver expression was only 0.75% of tumour levels. Expression in spleen was undetectable.

3.5. In vivo transfection and expression of IL-2 and IL-12

Mice (n = 5 per group) were administered with RTN:IL2/IL12 (25 µg of each plasmid encoding hIL-2 and mIL-12, 50 µg in total), or 50 µg of each plasmid (100 µg total). Tumours were excised at 24 h and minced tumours were cultured in medium. Cytokine levels were measured in the supernatants which were collected daily for three days. Production of both cytokines peaked at 24 h of culture with IL-12 at 76.81 and 90.32 pg/ml for 50 and 100 µg total dose, respectively and IL-2 at 28.40 and 45.65 pg/ml for 50 µg and 100 µg total dose, respectively (Fig. 3S) although the 100 µg dose was not significantly higher than the 50 µg dose.

3.6. Neuroblastoma growth retardation after i.v. administration of RTN:IL2/IL12

Neuroblastoma tumours were palpable 3 days after subcutaneous engraftment of Neuro-2A cells (1 × 10⁶) in the right posterior flank of A/J mice. On days 3, 5, 7, 9, 11, 13 and 15 post-engraftment, mice were injected with the RTN:IL2/IL12 (50 µg total plasmid), or with RTN:pCI (n = 15 per group), or glucose (n = 10). By day 11, 20% and 60% respectively, of mice receiving pCI or glucose had developed tumours of 12 mm or more and were killed, while all mice receiving RTN:IL2/IL12 had tumour diameters of less than 12 mm (p < 0.05) (Fig. 4A). By day 15, 54% of mice receiving RTN:pCI and 100% of mice receiving 5% glucose had developed larger tumours and were killed, while 87% of mice receiving therapeutic complexes had tumour diameters less than 12 mm (p < 0.05). After 90 days, five of 15 mice (34%) receiving therapeutic complexes and two of 15 mice (14%) receiving RTN:pCI still survived (Fig. 4A).

The average tumour volumes of mice receiving therapeutic complexes was reduced significantly by day 11 post-engraftment to 54 ± 85 mm³ compared with control mice receiving RTN:pCI or 5% glucose where the average tumour volumes were 215 ± 200 mm³ (p < 0.05) and 477 ± 249 mm³ (p < 0.001), respectively (Fig. 4B). For RTN:IL2/IL12, therefore, growth of the tumour at day 11 is retarded to about 11% of that in controls, while for RTN:pCI the figure is about 45%. Monitoring of individual tumours in each group over time illustrated the different kinetics of tumour growth (Fig. 4C), varying from rapid tumour growth to delayed tumour growth followed by later rapid growth, to complete eradication (Table 1). Delayed tumour growth (after 15 days) occurred in six mice from the group treated with IL2/IL12 while there were only two such mice in the plasmid control group and none in the glucose control group. Five mice displayed complete eradication in the treatment group compared to two in the plasmid control group and none in the glucose controls.

3.7. Leukocyte infiltration and reduced vascularization in tumours after treatment with RTN:IL2/IL12

Anti-CD45 staining showed that tumours from mice receiving RTN:IL2/IL12 had two to four times more leukocytes than tumours from mice receiving 5% glucose or RTN:pCI, respectively, p < 0.05 (Table 2). Moreover, tumours from mice receiving RTN:IL2/IL12 had 53 ± 9% of lymphocytes, five to thirteen fold more lymphocytes than were present in tumours from mice receiving glucose or RTN:pCI, respectively, p < 0.05. Control tumours had mainly monocytes and granulocytes.

Histological sections of tumours were stained with haematoxylin and eosin and examined for evidence of a therapeutic effect on the tumour such as extent of vascularization, and necrosis. Control tumours receiving 5% glucose were highly vascularized with dense capillary networks and large numbers of dividing cells (Fig. 5A and B). Tumours receiving RTN:pCI showed less extensive capillary networks and fewer dividing cells (Fig. 5C and D), and some necrotic areas (Fig. 5D), whereas in tumours receiving therapeutic complexes there was even less vascularization and more extensive necrotic areas, as defined by punctuate nuclear staining (Fig. 5E and F). Histological examinations of liver sections from controls (Fig. 5G), mice treated with pCI (data not shown) or with pCI-IL2/IL12 (Fig. 5H) showed that in all cases the liver parenchyma was normal, with no impairments in the cell lining of the remark cords, and no apparent lymphocyte infiltration or blood in sinusoidal areas.

3.8. Immunological memory against tumour cells in re-challenged mice

To establish if systemic immunological memory responses to tumour cells had been established, tumour-free mice (n = 2 for pCI controls and n = 5 for IL2/IL12) were re-challenged three months after the initial engraftment with 1.5 × 10⁹ wild-type Neuro-2A cells in the opposite flank to the original injection. All mice in both the pCI and IL2/IL12 groups remained tumour-free for at least 12 days, which was the point when all the control mice (n = 4) were killed due to their tumour size. The cellular anti-tumour immune response in re-challenged mice was assessed by isolating splenocytes for in vitro analysis at 12 days post-engraftment (Fig. 6). Splenocytes from wild-type mice that had received IL2/IL12 were co-cultured with Neuro-2A cells and producing significantly more spots than did splenocytes from control mice (p < 0.05). Mice that

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<td>Effects of IV injection of lipopolyplexes containing cytokine genes on leukocyte infiltration in tumours from mice.</td>
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A/J mice bearing s.c. tumour received seven i.v injections, two days apart, of 5% glucose, empty (ME42/DOPE:ME27:pCI) or therapeutic (ME42/DOPE:ME27:pCI-IL2/IL12) complexes. Results are presented as mean values ± SD (*p < 0.05 when leukocyte infiltration in tumours from therapeutic group was compared with the one from control groups).
Fig. 5. Tumour and liver histology. A/J mice bearing s.c. tumours were treated with 5% glucose (A, B), RTN:pCI (C, D) or RTN:IL2/IL12 (E, F) on days 3, 5, 7, 9, 11, 13, 15 post-engraftment, (n = 3 per group). Arrows denote blood vessels, stars denote dividing cells and (▲) indicates areas of necrosis. Representative sections are shown. Magnification ×40 for tumours. Representative liver sections of untreated control mice (G), and mice injected with RTN:pCILuc (H) are shown. Magnification ×200 for (C) and (H).
received RTN:pCI also produced higher numbers of spots but this was not significantly higher than controls.

4. Discussion

RTNs are nanoparticles comprising of lipid and peptide components for systemic gene delivery to tumours [3,4]. The RTN formulations target integrin receptors via an RGD peptide. They are shielded with “short PEG” (triethylene glycol) moieties providing stealth properties to reduce clearance from the circulation to reduce steric hindrance of peptide-mediated targeting and maintain good transfection efficiency. The RTN structure provides stability outside the cell and protects the DNA, but has the potential to disassemble within the cell in a step-wise process, in response to chemical and biological cues in the intracellular compartments. The aims of this study were to assess tumour delivery and transfection, biodistribution and therapeutic potential of RTNs in a murine model of neuroblastoma.

The synergy of interaction of liposomes, peptides and plasmid in the transfection process of the RTN was demonstrated in tumour transfection experiments where RTN:pCI-luc formulations resulted in twenty-five-fold higher luciferase expression than lipid formulations (ME42/DOPE:pCI-luc), and 100-fold higher than peptide complexes (ME27:pCI-luc) or pCI-luc DNA alone, consistent with previous cell culture experiments for similar RTN formulations [3,16]. Consistent with cellular studies, the integrin-targeted RTN, produced a 2.5-fold-higher level of transfected luciferase compared to a non-targeted formulation (ME42/DOPE:K16:pCI-luc). Thus the RTN is able to mimic intracellular pathogens such as Yersinia pseudotuberculosis, adenovirus and foot-and-mouth disease virus which all exploit integrins for cell targeting and entry [17]. Integrins are favoured receptor targets for tumour cells since the typical polarized, basolateral display of integrins breaks down, making them more accessible to targeting ligands, such as \( \alpha v \beta 3 \) expressed on tumour neovascular cells [18]. Integrins are expressed in neuroblastoma tumour cells including \( \alpha v \beta 3, \alpha v \beta 5 \) and \( \beta 1 \) integrins [19], all of which are targets for RGD peptide ligands and can be exploited as targets for receptor-mediated gene transfer vehicles. Biodistribution studies by qPCR showed a significant concentration of plasmid delivered by the RTN within the tumours, compared to lung and spleen and liver. Tumour specificity of RTN-mediated luciferase gene expression was even more pronounced, with at least 130-times more luciferase expression in tumours than in liver, lung or spleen. These results compare favourably with other studies where PEGylated nanoparticles demonstrated rapid uptake by the liver, spleen and lung in addition to tumour [20,21]. RTN administration produced no adverse pathology in the liver in contrast to a report with liposomal DNA formulations where liver accumulation was pronounced and was associated with significant hepatotoxicity [22]. Uptake to neuroblastoma tumours from the circulation is likely to involve the enhanced permeability and retention (EPR) effect [23] and the observed variability of transfection observed in quadrants of tumour is consistent with a role of the EPR effect where local differences in the tumour vascularization might be expected.

The in vivo transfection process from i.v. administration, based on this and previous studies with RTNs [3], might proceed as follows: firstly, the stealth coated RTNs persist in the circulation allowing them to penetrate the leaky tumour vasculature and accumulate within the tumour by the EPR effect. The RTN then engages with both integrin receptors and anionic cell-surface receptors of tumour cells, leading to cellular uptake. Environmentally responsive linkers allow RTN destabilization within the cell with the PEG removed from the ME42 lipid by esterase cleavage and the RGD targeting moiety of the peptide cleaved by furin or cathepsin B, releasing the nanoparticle from the integrins. The lipids ME42 and DOPE then mediate endosomal membrane fusion and cytoplasmic release of the plasmid DNA [24] while the remaining oligolysine peptide transfects the tumour with Luciferase gene expression.

Therapeutic studies with RTNs were conducted in the A/J mouse neuroblastoma model with genes for human IL-2 and a single-chain construct of murine IL-12, an effective cytokine combination in cell-mediated delivery studies [5,6]. Following injections of RTN:IL2/IL12 formulations, two thirds of mice experienced tumour eradication or retarded growth where the tumour size was stable during the series of injections and for a variable period of time afterwards, before expanding at an exponential growth rate whereas all untreated tumours grew exponentially from injection. Surviving mice without tumours were injected 3 months later by subcutaneous injection of viable Neuro-2A cells in the opposite flank to the original injection, and all mice survived whereas naïve controls grew exponentially as before, suggesting a vaccine effect of the original treatment. The cellular immune response in mice that survived re-challenge was assayed which indicated a cytotoxic T-cell response, consistent with results from previous cell-mediated delivery studies with IL-2 and IL-12 [5,6]. Tumours from animals treated with RTN:IL2/IL12, compared to control tumours, revealed a significant infiltration of leukocytes, particularly lymphocytes and reduced vascularization which may be explained by the angiostatic effects of IL-12 [25]. Necrosis of tumour tissue was increased, possibly due to a combination of the immune effector cells and the reduced vascular network, leading to a limited supply of nutrients and hypoxia.

In the control pCI treated group, 11 of 15 grew exponentially during the treatment phase, while, interestingly, two tumours were retarded and two were completely eradicated. Two surviving mice from the RTN:pCI group also survived re-challenge, but results from leukocyte infiltration and vascularization studies, in addition to splenocyte activation analysis from re-challenged mice, differed from those with the RTN:IL2/IL12 group suggesting some adjuvant, anti-tumour efficacy of the RTN:pCI complex itself. However, numbers at this stage are too small to draw firm conclusions and this mechanism for RTN:pCI requires further investigation. One possibility is that the repeated CpG motifs found in plasmids are immunostimulatory leading to inflammatory cytokine production which could augment the IL2/IL12 cytokine transgene expression [26].

We have previously demonstrated cytokine adjuvant immunotherapy with combinations of genes for IL-2 and IL-12, delivered by intratumoural injection of syngeneic tumour cells or fibroblasts [5,6]. Growth retardation of tumours after fibroblast:IL2/IL12 and RTN:IL2/IL12 delivery at days 11–12 were similar, at about 10% of
untreated tumours (5). Fibroblast delivery, however, longer term was more efficient with 90% of treated tumours eradicated compared to 33% for RTN:IL2/IL12. The in vivo, nanoparticle-mediated approach, however, offers advantages of a more conventional pharmaceutical approach to tumour therapy with the potential to target metastatic sites by systemic delivery, which could not be achieved by cell-mediated delivery approaches.

5. Conclusions

The RTN formulation is a nanoparticle delivery system with properties of stealth, receptor-targeting, synergistic lipid and peptide components and chemical linkers with environmentally-sensitive triggers to promote intracellular disassembly and high transfection efficiency. RTNs avoid clearance by first-pass organs, to specifically and efficiently deliver genes to tumours by systemic administration. Tumour transfection was significantly enhanced by incorporation of an integrin-targeting peptide ligand. We have illustrated the potential therapeutic efficacy of RTN formulations with IL2/IL12 cytokine genes. Tumour growth in most animals was significantly retarded and in one third, tumours were eradicated. RTN formulations may have potential for the generation of anti-cancer therapies, particularly for metastatic disease.

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Appendix

Figures with essential colour discrimination. Fig. 5 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.10.037.

Appendix. Supplementary material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.biomaterials.2010.10.037.

References