Development of adenoviral vectors armed with TNF-related therapeutic proteins for gene therapy

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Summary

Gene therapy has enthralled scientists in the last forty years due to its possibility of treating diseases at their genetic origin. The treatment is achieved by delivering genetic material into the patient cells via vectors (e.g., adenovirus).

Through the years, several gene therapies have been developed to treat monogenic diseases (e.g., spinal muscular atrophy [SMA], adenosine deaminase deficiency [ADA]), infectious diseases (e.g., HIV/AIDS, COVID-19), cancer, among others.

Fundamentally, cancer is a genetic disease that has apoptosis evasion and continuous proliferative signaling as main hallmarks. Over the years, the search for new cancer therapeutic proteins with lesser side effects than conventional chemotherapeutic drugs brought to the spotlight the apoptosis-inducing ligands from the tumor necrosis factor (TNF) family, such as TNF-α, FasL, and the TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is a protein that is able to selectively induces apoptosis in cancer cells while sparing the healthy cells. Another interesting member from the TNF family with potential therapeutic effects is the receptor activator of NF-κB ligand (RANKL). Together with its receptors RANK and OPG due to their involvement in cancer cell migration, including their possible association to fibrosis.

This thesis aims to develop adenoviral vectors armed with TRAIL and RANKL proteins for their use as treatments for cancer or fibrosis.

In **chapter two**, we reviewed the apoptosis-inducing ligands, TNF-α, FasL, and TRAIL, from the TNF superfamily as cancer therapeutics. We discuss how the receptor's specificity of the ligands has been improved by engineering mutants with higher affinity towards their DR than to their DcR, along with the development of DR-targeting antibodies. Moreover, consider the importance of reinforcing the ligands' trimeric conformation helps with the ligand's activity. More importantly, we discuss how the apoptosis-inducing ligands in fusion proteins and gene therapy (viral vectors) improve receptor activation and pharmacokinetics while, simultaneously, systemic toxicity can be avoided by localized administration and by using viral vectors with tumor-specific promoters.

In **chapter three**, we produced and successfully purified five different adenoviral vectors. Three of them are armed with a fusion protein containing a single-chain antibody against EGFR (scFv425) and one of the TRAIL variants (wild type TRAIL, DR4-specific [4C7], DR5-specific [DHER]). The other two vectors contain either RANKL WT or the RANKL_Q236D variant. The vectors were produced using the AdEasy system. They were purified using a two-step process: anion exchange chromatography, and ultrafiltration, giving high-purity vectors comparable with the standard CsCl method. In conclusion, this methodology is suitable for scale-up and allows using adenovirus vectors in *in vivo* studies.

EGFR is commonly overexpressed in diverse types of cancer, such as renal cell carcinoma (RCC), and plays a vital role in cell proliferation and tumorigenesis. In **chapter four**, we used CRISPR/Cas9 technology to knockout EGFR in RCC in combination with anti-cancer drugs (Gefitinib, Cetuximab, Cisplatin, Doxorubicin, Sunitinib) and TRAIL as a therapeutic option. The loss of EGFR in the RC21 cell line resulted in the inhibition of proliferation, cell arrest at the G2/M checkpoint, and increased resistance towards TRAIL. Additionally, we found that inhibition of PDGFR and VEGFR by sunitinib can attenuate the expression of pERK1/2 and pAKT induced by EGFR loss. In conclusion, using CRISPR/Cas9 technology to knock down overexpressed EGFR in combination with sunitinib may be a helpful in the course of treatment for RCC.

In **chapter five**, we tested the apoptotic activity of the adenoviral expressed fusion proteins scFv425-sTRAIL/DHER/4C7 from the vectors created in **chapter three** as a cancer treatment. The results showed that the fusion proteins reduced cell viability by apoptosis induction in cancer cell lines (COLO205, DLD-1, and RC21) while sparing the non-transformed cells (RPE-1) without any sign of toxicity. More importantly, the fusion proteins showed a higher apoptotic effect than the combined treatment of soluble TRAIL and anti-EGFR. Although we did not see a significant difference in the apoptotic activity between scFv425-sTRAIL, scFv425-DHER, and scFv425-4C7 in the tested cell lines, it is worth to continue exploring these fusion proteins.

In **chapter six**, we developed the Ad.RANKL WT and Ad. RANKL_Q236D to achieve long-term production and delivery of the RANKL proteins into fibrotic tissues. Fibrosis is characterized by an abnormal wound-healing response, leading to excessive accumulation of extracellular matrix (ECM). The secreted proteins were detected in the medium up to 19 days after adenoviral treatment of C10 cells with a maximum concentration of 30 nM; this corroborates the ability of the adenoviral vectors to achieve long-term delivery of a protein. Both RANKL proteins were functional, as was shown by their ability to activate RAW 264.7 macrophages. More importantly, RANKL_Q236D avoided inhibition by exogenous OPG and activated MMP9 gene expression. In conclusion, RANKL-armed adenovirus can be valuable tools for treating fibrosis.

In **chapter seven**, we summarize the findings of this thesis and point out some future perspectives of the described research. We discuss how adenoviral vectors can help deliver and induce a constant amount of therapeutic proteins, such as TRAIL and RANKL, thus overcoming their short half-life and avoid repeated administrations. We describe how the vectors can be modified to improve their specificity and efficacy. Furthermore, we allude to the CRISPR/Cas9 system as a gene engineering tool to study and develop specific treatments for cancer and other diseases.

Samenvatting

Gentherapie heeft de afgelopen veertig jaar veel aandacht gekregen van wetenschappers vanwege het feit dat je hiermee aandoeningen bij hun genetische oorsprong kan aanpakken. Behandeling met gentherapie begint met het zorgen dat stukjes genetisch materiaal in de cellen van de patiënt komen, dat kan via vectoren zoals een adenovirus. De afgelopen jaren zijn er verschillende gentherapieën ontwikkeld voor de behandeling van monogenetische aandoeningen zoals spinale musculaire atrofie (SMA) en adenosine deaminase deficiëntie (ADA), voor infectieziekten zoals acquired immune deficiency syndrome (Aids) en coronavirus disease (COVID-19), voor vormen van kanker en andere ziekten.

Kanker is een genetische aandoening waarbij cellen niet meer afsterven (apoptose) en zich blijven vermenigvuldigen. Er wordt veel onderzoek gedaan om therapieën te vinden die minder bijwerkingen hebben dan de conventionele chemokuren. Zo zijn er eiwittherapieën gevonden die apoptose induceren zodat de kankercellen wel afsterven, vaak zijn dit liganden die binden aan tumornecrosefactoren zoals TNF-α, FasL, en het TNF gerelateerde apoptose inducerende ligand (TRAIL). TRAIL is een eiwit dat heel selectief wel apoptose induceert in kankercellen maar niet in gezonde cellen, waardoor er mogelijk veel minder bijwerkingen zijn. Een ander potentieel zeer geschikt eiwit dat selectief apoptose kan induceren is de receptor activator van NF-κB ligand (RANKL) door te binden aan de receptoren RANK en Osteoprotegerin (OPG). Daarnaast is RANKL betrokken bij de migratie van kankercellen en waarschijnlijk ook bij fibrose.

In dit proefschrift hebben we geprobeerd om adenovirale vectoren te ontwikkelen met TRAIL en RANKL eiwitten om aandoeningen zoals kanker en fibrose te behandelen.

In **hoofdstuk twee** bediscussiëren we de mogelijkheden van de apoptose-inducerende liganden TNF-α, FasL en TRAIL, behorende tot de TNF superfamilie, voor kanker therapie. We behandelen hoe de specificiteit van de liganden voor receptoren aangepast en verbeterd is door verschillende mutanten te ontwikkelen met een hogere affiniteit voor de zogenaamde *death receptor* (DR) variant dan voor de zogenaamde *decoy* receptor (DcR) variant samen met het ontwikkelen van DR-specifieke antilichamen. De binding aan een DR leidt tot verhoogde celdood, apoptose, en binding aan de DcR, heeft geen effect. Daarnaast kan de binding van de liganden ook nog worden verhoogd door de trimeer conformatie van de ligand te stabiliseren. Bovendien bediscussiëren we hoe deze apoptose-inducerende liganden gefuseerd kunnen worden met andere eiwitten en via gentherapie (met virale vectoren) ingebracht kunnen worden. Dit leidt tot verbetering van receptor activatie en farmacokinetiek terwijl tegelijkertijd de bijwerkingen terug gedrongen kunnen worden door zeer gericht en lokaal toe te dienen en door het gebruik van virale vectoren met tumor-specifieke promotoren.

In **hoofdstuk drie** hebben we vijf verschillende adenovirale vectoren geproduceerd en gezuiverd. Drie hiervan zijn geladen met een fusie-eiwit met een enkele keten van een antilichaam tegen de *Epidermal Growth Factor Receptor* (EGFR) (scFv425) en met een van de TRAIL varianten (wild-type TRAIL of DR4-specifiek (4C7) of met DR5-specifiek (DHER)). De andere twee vectoren bevatten dan wel wild-type RANKL of de RANKL_Q236D mutant. De vectoren zijn gezuiverd via een twee-stap procedure namelijk; anionenuitwisselingschromatografie en ultra-filtratie. Dit resulteerde in zeer zuivere vectoren met een vergelijkbare zuiverheid van de vaak gebruikte CsCl methode. De conclusie is dat de beschreven methode bruikbaar is voor schaalvergroting en het gebruik van adenovirale vectoren voor *in vivo* studies mogelijk maakt.

EGFR wordt vaak tot overexpressie gebracht in verschillende typen van kanker, onder andere in niercelkanker (RCC), en speelt een belangrijke rol in ongeremde cel groei en bij het ontstaan van tumoren. In **hoofdstuk 4** hebben we CRISPR/Cas9 techniek gebruikt om het EGFR-gen uit te schakelen (knock-out) in RCC cellen, dit hebben we gecombineerd met anti-kanker medicijnen (Gefitinib, Cetuximab, Cisplatina, Doxorubicine, Sunitinib) en met TRAIL, als een therapeutische optie. Het verlies van het EGFR-gen in de RC21 cellijn leidde tot inhibitie van de groei van de cellen, ze bleven in hangen in het G2/M controle punt van de celcyclus, en deze cellen hadden een verhoogde resistentie tegen TRAIL. We hebben ook gezien dat de remming van PDGFR en VEGFR door Sunitinib de expressie van pERK1/2 en pAKT verlaagd. Deze pERK1/2 en pAKT waren juist verhoogd door het verlies van EGFR. We concludeerden dat het gebruik van de CRISPR/Cas9 techniek om het verhoogde EGFR uit te schakelen in combinatie met Sunitinib zeer bruikbaar kan zijn in de behandeling van niercelkanker.

In **hoofdstuk vijf** hebben we de apoptose inducerende fusie-eiwitten scFv425-sTRAIL/DHER/4C7 tot expressie gebracht via de adenovirale vectoren van **hoofdstuk drie** en getest als anti-kanker behandeling. Het resultaat was dat verschillende kankercellijnen (COLO205, DLD-1, and RC21) een verlaagde levensvatbaarheid hadden, en dat niet getransformeerde cellen (RPE-1) geen

enkele toxiciteit lieten zien. Bovendien lieten de fusie-eiwitten een hoger apoptotisch effect zien dan de gecombineerde behandeling met de oplosbare variant van TRAIL en de uitschakeling van EGFR samen. Er was geen significant verschil tussen de scFv425-sTRAIL, scFv425-DHER, en scFv425-4C7 onderling in de door ons geteste cellijnen, en het is zeker interessant om deze fusie-eiwitten verder te bestuderen.

In **hoofdstuk 6** hebben we adenovirale vectoren ontwikkeld die RANKL of de meer specifieke mutant RANKL_Q236Dtot expressie kunnen brengen. Dit om lange termijn productie en aflevering van RANKL eiwitten aan fibrose weefsel te bereiken. Fibrose wordt gekarakteriseerd door een ongewoon wondgenezing proces, waardoor er teveel extracellulaire matrix (ECM) ontstaat. De uitgescheiden RANKL eiwitten konden tot 19 dagen na toediening van de adenovirale vectoren gedetecteerd worden in het medium van C10 cellen, tot een maximum concentratie van 30 nM. Dit bevestigd de mogelijkheid van adenovirale vectoren om een lange termijn afgifte van eiwitten te verzorgen. Allebei de gebruikte varianten van RANKL waren functioneel, want ze konden RAW 264.7 macrofagen activeren. Bovendien had RANKL_Q236D geen last van inhibitie door extra toegevoegd OPG en activeerde het expressie van het matrixmetalloprotease (MMP9)-gen. In conclusie; RANKL geladen adenovirussen kunnen een bruikbare methode worden om fibrose te behandelen.

In **hoofdstuk 7** worden de resultaten uit dit proefschrift samengevat en worden er een aantal toekomstperspectieven besproken. We bediscussiëren hoe adenovirale vectoren kunnen bijdragen aan het afleveren en induceren van een constante hoeveelheid therapeutische eiwitten zoals TRAIL en RANKL. Met deze vorm van toediening levert de snelle verwijdering van het therapeutische eiwit uit het lichaam geen probleem op en kan het frequent toedienen van dit eiwit voorkomen worden. We beschrijven hoe vectoren aangepast kunnen worden om hun specificiteit en werkzaamheid te verhogen. Daarna beschrijven we op hoe de CRISPR/Cas9 techniek gebruikt kan worden als genetisch hulpmiddel om nieuwe specifieke behandelingen voor kanker en andere aandoeningen te ontwikkelen en te bestuderen.

Appendix

Acknowledgments

About the author

List of publications

A

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About the author

Olivia was born in San Cristóbal de las Casas, Chiapas, Mexico on August 25th, 1988. In 2011, she obtained her bachelor's degree in Chemical and Pharmaceutical Biology with an emphasis in pharmacy at the Universidad Veracruzana. For her bachelor thesis, she studied the effect of diazepam and flavonoid chrysin on aggressive behavior in *Betta splendens* and its relation to anxiety. In 2014, Olivia obtained her master's degree in Biotechnology at the Universidad de Las Americas Puebla. In there, she cloned, expressed, and characterized a microbial lysyl oxidase (LOX) for her thesis.

In 2015, She obtained a PhD research fellowship from the Mexican National Council of Science and Technology (CONACyT). Consequently, she moved to The Netherlands to pursue her doctoral degree in the Department of Chemical and Pharmaceutical Biology at the University of Groningen, under the supervision of Prof. dr. Hidde J. Haisma. Her postgraduate studies were focused on the development of adenoviral vectors for gene therapy.

Lists of publications

Liu, B., Diaz Arguello, O. A.*, Chen, D., Chen*, S., Saber, A., & Haisma, H. J. (2020). CRISPR-mediated ablation of overexpressed EGFR in combination with sunitinib significantly suppresses renal cell carcinoma proliferation. *PLoS ONE*, *15*(5), 1–13. https://doi.org/10.1371/journal.pone.0232985

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Wang, Y. *, Diaz Arguello, O. A. *, Setroikromo, R., Suo, F., Haisma, H. J., & Quax, W. j. Long term secretion of adenovirally expressed RANKL WT/ Q236D for treatment of fibrosis

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