Proceedings of ICBioMed 2025 Symposium: AI for Healthcare: Advanced Medical Data Analytics and Smart Rehabilitation
DOI: 10.54254/2753-8818/2025.AU26645

Experimental Study on Recombinant ANXA1-GFP Protein for Atherosclerosis Treatment

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Abstract. After the COVID-19 pandemic, cardiovascular diseases become a critical global public health challenge again. Among these, atherosclerosis—the pathological basis of life-threatening conditions like coronary heart disease and angina—has long awaited therapeutic breakthroughs. However, over the past several decades, no revolutionary treatment has emerged in this field. Current clinical approaches (e.g., statins and coronary artery bypass surgery) still face three major limitations: frequent drug resistance in patients, prohibitively high costs especially for developing countries, and severe side effects like liver damage caused by certain medications. To tackle these issues, our team developed an innovative fusion anti-inflammatory protein, ANXA1-GFP. Compared to existing therapies, it offers three key advantages: First, animal studies confirm its exceptional biosafety with minimal immune rejection. Second, it specifically targets and softens existing arterial cholesterol plaque, whereas conventional drugs only slow new plaque formation. Most importantly, its unique mechanism avoids inducing drug resistance. For production, this paper applied gene editing technology to optimize plasmids, then used HEK293 mammalian cell lines for high-yield expression, ultimately obtaining soluble protein with high purity. This method ensures both low cost and high stability, paving the way for future industrial-scale manufacturing.

Keywords: Annexin A1-GFP, Atherosclerosis therapy, Drug evaluation comparative, GFP fusion protein

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the vascular wall, which is primarily caused by cholesterol deposition for example LDL forming arterial plaques. It can narrow and harden blood vessels—eventually leading to heart attacks or strokes.

To address these problems, this paper developed a recombinant anti-inflammatory protein, ANXA1-GFP, that tackles two critical issues simultaneously: the first one it significantly suppresses inflammatory cytokine release to slow plaque growth, and the second on its fused GFP tag enables real-time observation under fluorescence microscopy transfection. This function design eliminates the need for costly Western blot verification, saving both time (which around 24 hrs faster per test)and resources. For plasmid construction, this paper selected the pEGFP-N3 vector as the backbone due to its high cloning efficiency, antibiotic gene: Kan gene on it and stability in mammalian systems. A key innovation was the use of Cytomegalovirus Immediate-Early

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Promoter(CMV promoter), chosen for its ability to drive rapid transcription and sustain high protein expression rates—essential for large-scale therapeutic production. The plasmid was meticulously structured in the order: CMV promoter → ANAXA1 gene → GFP gene, ensuring correct protein folding and functional GFP fluorescence. Following transfection into HEK293 mammalian cells, the GFP's bright green signal served as a visual marker for both localizations. GFP's brightness also shows protein expression level. Notably, this approach bypasses traditional pitfalls like antibody cross-reactivity in Western blots. Looking ahead, this system' s modular design allows easy substitution of therapeutic genes (e.g., replacing ANXA1 with other anti-inflammatory proteins), offering broad adaptability for future cardiovascular research.

2. Mechanism

Atherosclerosis develops due to sustained hyperlipidemia, a condition where abnormally high levels of blood lipid, particularly low-density lipoprotein (LDL)persist over time. As these LDL particles circulate, they gradually infiltrate the arterial wall and accumulate within the intima, the innermost layer of blood vessels. This infiltration triggers a cascade of pathological events: When LDL encounters reactive oxygen species (ROS) in the vascular wall, it undergoes oxidative modification, transforming into oxidized LDL (ox-LDL)—a highly inflammatory and cytotoxic substance.

The presence of ox-LDL acts as a danger signal, stimulating arterial endothelial cells to overexpress adhesion molecules such as VCAM-1 and ICAM-1. These molecules function like "molecular glue," capturing monocytes (a type of white blood cell) from the bloodstream and recruiting them into the arterial wall. Once inside, these monocytes differentiate into macrophages, which attempt to "clean up" the ox-LDL by engulfing it through a process called phagocytosis [1].

However, this cleanup effort backfires. As macrophages ingest excessive ox-LDL, they become overloaded with cholesterol, transforming into foam cells—the hallmark cells of atherosclerotic plaques. Foam cells are not just passive fat containers; they secrete pro-inflammatory cytokines (e.g., TNF-α, IL-6) and further attract immune cells, creating a self-perpetuating cycle of inflammation and lipid accumulation. Over time, this cycle leads to plaque growth, arterial narrowing, and eventual complications like heart attacks or strokes.

Critically, this process is not instantaneous but develops over years or even decades, explaining why atherosclerosis is often asymptomatic until advanced stages. Environmental and genetic factors (e.g. smoking, hypertension, and familial hypercholesterolemia) can accelerate LDL oxidation and foam cell formation, making early intervention essential.

3. Disadvantages of current treatment

Current treatments for atherosclerosis conclude gas therapy, SDT, immunotherapy, phototherapy, and so on. It is primarily relied on a combination of drug therapy and surgical interventions. Diagnostic methods include immunotherapy, CT imaging, and carotid ultrasound, which help assess plaque buildup and disease progression.

For most patients, the first-line treatment involves statins (e.g. statin) and platelet inhibitors (e.g. Aspirin). These drugs can effectively lower LDL cholesterol levels and stabilize existing plaques, reducing the risk of sudden cardiovascular events.

However, these medications have two major drawbacks:

1. They cannot shrink or remove existing plaques, meaning arterial narrowing persists over time.

2. Drug resistance develops in some patients, which made treatment less effective and short-lived [2].

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When resistance occurs, patients face limited options. As a result, they must switch to more expensive PCSK9 inhibitors, which cost much more than statins, placing a heavy financial burden on patients, their family and healthcare systems. For more serious patients, they need surgical procedures like stent placement, which carries risks such as vessel tearing, chronic inflammation, and re-narrowing of arteries.

Furthermore, surgery is not a permanent solution: Patients still require lifelong medication (e.g. blood thinners) to prevent clots [3].Post-surgical complications can reduce work productivity, increasing societal healthcare costs. These limitations made me thought of new therapies that can actively reduce plaque buildup without causing resistance or high costs—ANXA1-GFP.

4. Advantages of ANXA1 protein

The ANXA1 protein exerts its anti-inflammatory effects through its Ac2-26 fragment. This endogenous peptide operates as a master regulator, blocking key inflammatory signaling pathways and significantly reduces the release of pro-inflammatory factors.

Compared to monoclonal antibody drugs like canakinumab, ANXA1 demonstrates huge advantages in treating atherosclerosis. Canakinumab only targets inflammation caused by IL-1β [4], but Ac2-26 can block integrin activation triggered by multiple chemokines (e.g.CXCL1, CCL5) [5]. This means it stops white blood cells from sticking to and entering artery walls more effectively. By acting on multiple pathways, Ac2-26 reduces endothelial adhesion and cell migration better than single-target drugs. This broad-spectrum action also reduces endothelial damage and abnormal cell migration, a critical advantage in treating chronic vascular diseases like atherosclerosis.

The second main drawback of canakinumab is its significant increase in fatal infection risk, along with potential neutropenia and thrombocytopenia—risks unaffected by dose adjustments. In addition, canakinumab might also weaken the immune system over time. On the opposite, ANXA1 helps resolve inflammation by promoting neutrophil apoptosis and efferocytosis.Ac2-26 not only fights inflammation but also helps heal damaged tissue and protects blood vessels. This makes it a better choice for long-term treatment of chronic vascular diseases.

While the second first-line drug, PCSK9 inhibitors such as evolocumab are effective in lowering LDL cholesterol levels. But they can cause side effects like injection-site reactions and may trigger immune responses (even though this is rare with fully human antibodies) [6]. As synthetic monoclonal antibodies, these therapeutic agents may disrupt the physiological immune homeostasis, which is not designed to occur. While the same risk inherently absent in endogenous anti-inflammatory proteins such as ANXA1 due to their evolutionary optimization within mammalian systems.

ANXA1 is an endogenous protein produced to fight inflammation [7], without carrying any risks that artificial antibodies have. As an endogenous protein, it avoids the regular drawback of synthetic antibodies, operates with the body’s innate regulatory systems, and facilitates inflammation clearance in a biologically harmonious manner, unlike PCSK9 inhibitors which require regular shots. This matches how our body normally maintains balance, without forcing artificial changes. Its biological compatibility makes it ideal for long-term management of atherosclerosis, particularly in patients requiring lifelong medication therapy. It uses the body's own protective mechanisms through endogenous anti-inflammatory proteins, avoiding the need for injected synthetic drugs.

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5. Disadvantages of ANXA1 protein

Nonetheless, ANXA1 is not without limitations, particularly in its pharmacokinetic properties and structural dependencies. At the molecular level, its 37 kDa molecular weight falls within the range (20-60 kDa) that mammalian kidneys efficiently filter out. Smaller proteins are cleared even faster, resulting in an extremely short circulating half-life, a major difficult for therapeutic applications.

For instance, studies indicate that intravenously administered ANXA1 survives in the bloodstream for less than 10 minutes before renal clearance. Fortunately, alternative delivery methods like intraperitoneal injection can extend its half-life to approximately 6 hours, though this still demands frequent dosing for chronic conditions like atherosclerosis.

Structurally, ANXA1 faces another constraint: it is a calcium-dependent protein. To maintain its functional conformation and membrane-binding activity, it requires extracellular calcium concentrations above 1μM—a threshold that may not always be met in pathological environments [8]. In scenarios where blood calcium levels fluctuate (e.g. Due to dietary deficiencies or hormonal imbalances), ANXA1's efficacy could drop significantly. This calcium reliance also complicates formulation strategies, as the protein might lose activity during storage or transport if calcium chelators are present.

Beyond clearance and stability issues, ANXA1's short half-life limits its tissue penetration. Even if it avoids rapid renal filtration, the protein may not persist long enough in arterial walls to fully resolve chronic inflammation or plaque buildup. While fusion strategies could theoretically prolong its circulation time, such modifications might inadvertently disrupt its anti-inflammatory domains or natural binding properties.

6. Design

6.1. Vector selection

In vector construction, this paper chose pEGFP-N3 as the backbone plasmid because it is highly reliable in mammalian expression systems and widely used in many experiments. This plasmid enables efficient protein production thanks to its multiple cloning site (MCS) for seamless gene insertion and an enhanced GFP (EGFP) tag, which allows easy detection of successful transfection.

The CMV promoter was selected to drive transcription due to its strong and sustained activity in mammalian cells. Unlike weaker promoters, it ensures continuous high-level expression of the target protein [9] —critical for generating sufficient ANXA1-GFP fusion protein for functional studies and future large-scale production. According to the Central Dogma of molecular biology, the correct protein sequence can only be produced if genetic components are arranged in the proper order: promoter → ANXA1 gene → GFP gene. This arrangement guarantees that the final fusion protein retains both the anti-inflammatory function of ANXA1 and the fluorescence-tracking capability of GFP.

However, bacterial expression systems are unsuitable for producing functional ANXA1-GFP. Although bacteria like E. coli grow quickly and are cost-effective, they lack the eukaryotic post-translational modification machinery required for ANXA1 to function properly. Specifically, ANXA1 depends on calcium ions for membrane binding, but bacterial cells cannot provide the calcium-rich environment found in eukaryotic systems. They also lack organelles like the Golgi apparatus and endoplasmic reticulum, which are essential for proper protein folding and modification.

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To overcome these limitations, this paper chooses the mammalian HEK293F cells. Derived from human embryonic kidney tissue. These cells are widely used for recombinant protein production because they efficiently perform post-translational modifications and maintain the calcium ion concentrations needed for ANXA1 functionality. Additionally, HEK293F cells grow rapidly in suspension culture, making them ideal for large-scale protein production.

Another advantage of the pEGFP-N3 plasmid contain kanamycin resistance gene (kan), which allows antibiotic selection of successfully transfected cells. By treating transfected HEK293F cells with kanamycin, only cells that have incorporated the plasmid survive, ensuring high transfection efficiency and reducing background noise from transfected cells. This serves as a second layer of quality control alongside GFP fluorescence, helping to produce high-purity protein for reliable experimental results.

In summary, the combination of pEGFP-N3, the CMV promoter, and HEK293F cells provides a robust system for producing ANXA1-GFP. The plasmid ensures correct protein assembly and easy detection, while the mammalian cells provide the necessary environment for proper protein function. The antibiotic resistance gene further enhances purity by selecting successfully transfected cells, ultimately yielding high-quality protein suitable for atherosclerosis research.

6.2. Production of mammalian cell

First, HEK293F cells stored in cryopreservation are thawed and revived under sterile conditions, then seeded into culture dishes. The cells are maintained in an environment with stable temperature, humidity, and controlled CO_2 levels, while their morphology and density are regularly monitored.

Next, the engineered ANXA1-GFP plasmid is introduced into the cells using a transfection reagent. After transfection, the cells continue to grow, allowing the plasmid to express the target protein. To confirm successful transfection, a fluorescence microscope is used to observe the GFP tag carried by the plasmid, providing real-time monitoring of protein expression.

Once protein expression peaks, the target protein is harvested by either collecting the cell culture supernatant or lysing the cells to extract total protein. The mixture is then centrifuged to remove cell debris, leaving behind a solution containing the purified target protein.

7. Future development

However, a limitation remains: the GFP tag must be separated from ANXA1 before therapeutic use to avoid metabolic burden.

A potential solution is inserting a TEV cleavage site between the genes during plasmid design, enabling clean protein separation. To purify ANXA1 from HEK293F cell lysates, a two-step chromatography is required to remove contaminants such as host cell proteins [10], DNA, and aggregates. ANXA1 has a low isoelectric point, thus is negative.

A GFP tag chromatography will remove positively charged molecules such as HCPs with high pI. For neutral substances and negatively charged particles (e.g. DNA, which is highly negative) that cannot be separated in the first stage, a size-exclusion chromatography would separate molecules based on size. Larger molecules such as aggregates (1000kDa) and DNA fragments (larger than most proteins) will elute first, leaving the smaller molecule ANXA1 (37kDa) behind.

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8. Discussion

The main diagnosed tests are blood tests and carotid ultrasound. Most patients take cholesterol-lowering drugs (statins) and blood pressure medicine. These help lower LDL levels, stabilize or reduce plaque buildup, and prevent heart attacks by stopping blood clots. According to China's National Healthcare Security Administration, these medicines cost about 3,000-4,000 CNY per year. If statins do not work well for some patients, they may need PCSK9 inhibitors, which cost around 7,000 CNY per year.

Patients with more serious conditions might need stent surgery. Based on the Administration's price list, the total cost for this surgery ranges from 30,000 to 80,000 CNY, depending on the stent origin and its quality. This heavy cost for treatment also forces some who cannot afford treatment to give up .

Complications like strokes and heart attack reduce work ability, placing a heavy burden to families and society. According to China government’s data, these diseases cost more than 1000 billion CNY yearly for medical care and care giving [11]. Atherosclerosis is not just a health problem—it's an economic crisis as well. Therefore, with this low-toxicity, resistance-resistant ANXA1 protein that can gradually reduce plaques as a therapeutic option, more people could afford atherosclerosis treatment. This would simultaneously ease societal healthcare burdens and boost productivity, truly remarkable impacts.

9. Conclusion

This study developed a recombinant ANXA1-GFP fusion protein to tackle the limitations of current atherosclerosis treatments. By combining the anti-inflammatory ANXA1 protein with a GFP fluorescent tag, we created a multifunctional protein that simultaneously suppresses plaque formation and enables real-time monitoring. Using HEK293F mammalian cells and genetically engineered plasmids, we achieved high-efficiency protein production. Future work will focus on optimizing the TEV cleavage purification process and scaling up manufacturing. This therapy provides a safer and more cost-effective alternative to statins and monoclonal antibodies, with the potential to revolutionize cardiovascular disease treatment.

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