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Review Article

Co-culture Methods Used to Model Atherosclerosis In Vitro Using Endothelial, Smooth Muscle and Monocyte Cells

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Abstract

Atherosclerosis is a progressive, chronic, inflammatory disease that causes the narrowing and hardening of both the medium and large sized arteries by excessive plaque formation within the artery walls. It is expected that by 2020, atherosclerosis will be the leading cause of death globally. The mechanisms of atherosclerosis are complex and multivariate, not all of which are fully understood. The use of *in vitro* models is vital to mechanistically predict the main triggers of the disease, disease risk level, determine suitable treatments and to ascertain the efficacy of potential treatment options. This review provides a comprehensive summary of the different studies and co-culture methods used to investigate the mechanisms of atherosclerosis *in vitro*.

Introduction

Atherosclerosis is the gradual constriction of arteries, brought about by plaque formation within the artery walls [1,2]. Atherosclerosis has a long asymptomatic phase, which tends to be further aggravated by other existing conditions, such as diabetes [3] and hypertension [4-6]. Later stages of atherosclerosis are characterized by the formation of unstable plaques, which are vulnerable to rupture and thus initiate the coagulation cascade, leading to thrombus formation and potentially embolism [7,8]. Thrombosis is the local clotting of blood within the circulatory system, while embolism is the lodging of detached intravascular mass–in this case a blood clot–in a vascular vessel [9]. These two events are precursors to more serious cardiovascular diseases and clinical cardiac events such as Myocardial Infarction (MI) and stroke [2]. Atherosclerosis is a prevalent cause of morbidity and mortality in developed countries [10]. However the incidence of this disease is also increasing in developing countries and it is predicted that, by 2020, atherosclerosis will be the leading cause of death globally [11].

The mechanism by which atherosclerosis is brought about is an extremely complex process involving both biochemical and cellular events [12]. The early events of atherosclerosis occur as a result of injury to the endothelial layer of the vascular wall, which can be triggered by elevated blood pressure or cholesterol [13] (Figure 1A and 1B). This in turn activates monocytes and drives their recruitment to the dysfunctional endothelium (Figure 1C). Research is underway to determine the exact triggers that initiate the onset of atherosclerosis [14,15]. It is hypothesized that toxins from tobacco usage, diabetes, elevated blood pressure or dyslipidemia can lead to irritation and damage of the endothelium. Consequently, permeability of Low-Density Lipoproteins (LDLs) into the intima, or sub-endothelial region, increases. Once inside the intima, LDLs undergo oxidation, which triggers Endothelial Cells (EC) to express cell adhesion molecules such as Vascular Cell Adhesion Molecule-1 (VCAM-1) on their membranes, and simultaneously to secrete chemokines. These secreted chemokines act as signaling cues that attract leukocytes which transmigrate across the blood endothelium [16], while adhesion molecules ensure the arrest of monocytes and T-cells (subsets of leukocytes) to the arterial wall.

Through activation of signaling mechanisms, there is an upregulation of cell adhesion proteins on the activated endothelium as well as on the monocytes as a downstream effect. It is well established that adhesion proteins (L-selectin, P-selectin and PECAM-1) bring blood-suspended monocytes to a tether that rolls along the endothelial surface before endothelial adhesion proteins (ICAM-1 and VCAM-1) completely arrest the immune cells [17]. The final stage here is diapedesis of the monocytes, resulting in their accumulation within the endothelium [18,19]. Localized monocyte activation, transendothelial migration and differentiation within the endothelium are influenced by Macrophage Chemotactic Protein-1 (MCP-1) and macrophage Migratory Inhibitory

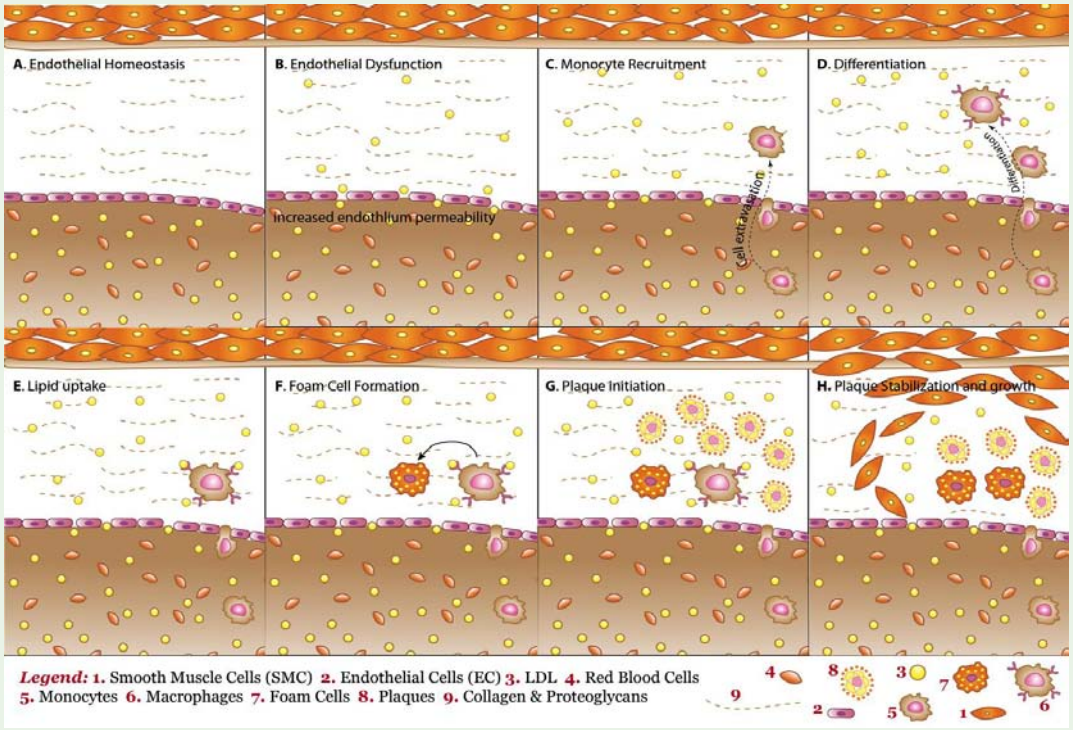

Figure 1: Accepted sequence for the initiation and progression of atherosclerosis. (A and B) Pathophysiological dysfunction of an otherwise healthy endothelium causes a localized increase in blood vessel permeability and (C) initiation of monocyte recruitment cascade. (C and D) After translocation into the adjacent interstitial space monocyte differentiate into macrophages and (E) proceed with uptake of lipids that also leak into the same compartment. (F) Progressive uptake causes the macrophage to be filled and have foam like content. All this time the localized inflammatory response is intensified and recruiting smooth muscle cells from the intima towards the sites of inflammation. The accumulation of lipids, cells and plaque all contribute towards a growing lesion resulting in a narrowing of the lumen.

Factor (MIF) [15], a chemokine produced at the site of injury. MCP-1 deficient mice exhibited attenuated atherosclerosis, indicating the key role of monocytes in disease progression [13]. After the monocytes transmigrate across the endothelial layer, they differentiate into macrophages (Figure 1D). Differentiated macrophages begin to engulf lipids through phagocytosis [14,15] (Figure 1E), and break them down into cholesteryl esters, which form foam-like deposits. Afterwards, cholesteryl esters are broken down into free cholesterol and fatty acids. At this stage, macrophages are known as foam cells due to their lipid-rich foamy-like contents [20] (Figure 1F). These processes are further intensified by an inflammatory responses [14,15].

Endothelial homeostasis is regulated by the endothelium itself, as it releases anti-thrombotic factors that hinder platelet adhesion and aggregation countered by pro-thrombotic release of mediators that encourage platelet activation [21]. Platelet activation not only further encourages a thrombotic environment but it leads to the increase accumulation of Platelet Derived Growth Factor (PDGF), and Lysophosphatic Acid (LPA) which stimulates the migration and proliferation of Smooth Muscle Cells (SMC) from the medial (inner) into the intimal (middle) layer of the vessel wall [22,23]. Endothelial, smooth muscle and macrophage cells at sites of disrupted intima and responding to the various inflammatory cues all amass lipids, through passive and active cell membrane translocation, to form foam cells, resulting in the creation of atherosclerotic plaques [24,25] (Figure 1G). The formation of foam cells is exacerbated by oxidative stress which results in further lipid oxidation [26]. The lipid accumulation alongside the increased proliferation of SMC within the intima creates a growing lesion that radially pushes towards the lumen to cause a decrease in the luminal diameter of the artery and also brings about arterial hardening [27] (Figure 1H).

Urgent Need for Unified In Vitro Models

The *in vivo* mechanisms of atherosclerosis are complex, multivariate, and not fully understood [12]. It is vital to increase understanding of the mechanistic progression of atherosclerosis so that improved treatment options can be devised. Animal models have been employed to study disease development. These models are also used prior to clinical trials in order to screen and determine the suitability of various drug candidates as potential treatments [28]. However, the way atherosclerosis manifests within various animal models differs, which prevents representative studies from being carried out [28]. There is evidence to suggest that the response to drugs within animal models can even be influenced by the time of animal sacrifice as well as variations between the sites of lesions tested [29]. Biological differences between species can yield spurious results; that a drug appearing to show positive results in the murine model fails once it reaches clinical trials is a recurring scenario. The efficacy of a candidate drug may be highly dependent on the presence or absence of specific proteins in the corresponding test subjects, and this can only be determined in a clinical setting [30-32]. Unsuitable drug candidates that make their way into clinical trials are unnecessary and ultimately harmful to the patient, expensive, and time consuming.

It is thus necessary to create models that accurately reflect the physiology of atherosclerosis. Appropriate, representative testing with detailed *in vitro* atherosclerosis models can facilitate prediction of the main triggers of the disease, estimate disease risk level, determine suitable treatments and ascertain the efficacy of potential treatment options [28]. The use of cell co-cultures allows the target drug identification process for atherosclerosis to be carried out under a range of physiological conditions so that changes in the lead molecule activity in response to alterations in these conditions can be ascertained. These models are more representative of the *in vivo* environment, allowing both normal and diseased conditions to be approximated [33].

Aside from using co-culture based models to study the pathogenesis atherosclerosis, such *in vitro* models are used to evaluate new technologies for treatment by determining the biocompatibility of new stents / bio-engineered implants such as vessel prototypes [34,35]. Additionally, delivery methods for plasmid DNA (pDNA) [36] and nanoparticles [37,38] for specific targeting of vasculature can be assessed. Better insight into susceptibility to atherosclerosis in patients with pre-existing conditions such as obesity [39], diabetes [3,40-43] and HIV [44] has been gained using *in vitro* models. Drug testing both for existing medications such as statins [45-49], as well as novel, re-purposed and alternative medicines [50-54] is being carried out using *in vitro* artery models. Furthermore, the atheroprotective effect of dietary active ingredients [55,56] and diets such as the Mediterranean diet [57,58], are also being gauged using *in vitro* models.

Despite their extensive use, there is no convergence of arterial *in vitro* models that facilitates multi-institutional comparison of results to facilitate significant progression in atherosclerosis science. From here on, this review focuses on the biological make up and summarizes current multi-culture approaches to mimic the arterial wall on the bench. Cell types, Extracellular Matrix (ECM) components, biomechanical forces, biochemical cues, and geometry of cell culture receptacle are factors that should be considered for recreating accurate *in vitro* models.

Role of Vascular Wall Cell Types in Atherosclerosis

The surfaces of blood vessels within the circulatory system are comprised of three main layers; the adventitia, media and intima. The intima is the innermost layer of the blood vessel which is composed of EC; this layer forms the lumen of the vessel, which is in continuous contact with the blood. Between the endothelium and the media is a layer of basement membrane proteins, predominantly laminin and collagen IV. This is followed by a layer of elastin. The primary constituent of the media layer is SMC embedded in a network of connective tissue. This layer regulates blood flow and pressure through vasoconstriction and vasodilation via SMC contraction. The adventitia is the outermost layer of the blood vessel and is made up of loose connective tissue and fibroblasts [33].

The endothelium forms a dynamic layer with the blood, and acts as a barrier between the blood and the surrounding tissues. Additionally, it prevents blood clot formation and leukocyte extravasation when it is in a functional state [59]. Different parts of the circulatory system appear to be either resistant to atherosclerosis (atheroprotective) or prone to atherosclerosis (atheroprone) [60]. Unbranched regions of the cardiovascular network tend to be atheroprotective in nature. Therefore, they are predisposed structurally, biochemically and functionally to resist atherosclerosis. Atheroprotective EC has ellipsoidal cell morphology and aligns them in the primary direction of flow. The steady production of Nitric Oxide (NO) from Nitric Oxide Synthase (eNOS) is important for vascular homeostasis, is anti-atherogenic and is hypothesized to regulate monocyte adhesion [61]. Atherosclerosis often occurs in particular "lesion-prone" areas of the circulatory system. These tend to be where blood vessels branch or curve, creating areas of low time-average shear stress. EC from atheroprone regions tend to have a dysfunctional phenotype. These cells are pro-thrombotic, pro-inflammatory, and have impaired barrier function. They also display a cuboidal morphology, have high cellular turnover, senescence rates and are predisposed to retaining lipoproteins. Cardiovascular risk factors such as hyperlipidemia, oxidative stress and smoking are mediators for endothelial dysfunction.

EC activation is identified by the cytokine induced expression of surface adhesion proteins [62]. This facilitates the recruitment of leukocytes circulating in the blood stream. Mediators of endothelial activation are Advanced Glycation End-Products (AGEs), disturbed blood flow, and pro-inflammatory cytokines, which act through the activation of the transcription factor NF-κB [63]. EC activation can be inhibited through the action of NO. Endothelial dysfunction is worsened by EC activation.

SMC within the medial layer of the blood vessel are involved in maintaining the vessel diameter in response to external stimuli. SMC have three main phenotypes both *in vivo* and *in vitro*: synthetic, contractile and quiescent. SMC in a healthy vessel exist in the contractile phenotype where they conduct myogenic autoregulation according to changes in blood pressure or flow. However, in an atherosclerotic environment SMC undergo phenotypic switching from the contractile into the proliferative state. In this state, the myogenic autoregulation function of the SMC becomes impaired and they begin to proliferate and migrate from the medial layer into the intimal layer through both cytoskeletal and ECM remodeling. This process leads to a local thickening of the vessel wall and a decrease in vessel diameter, potentially compromising blood flow. During atherosclerotic plaque formation, Matrix Metalloproteinase (MMP) is produced within the lesions. MMPs contribute to ECM remodeling to enable SMC to proliferate and migrate more within the lesion. The SMC become a major component of the atheroma and form a fibrous cap around it, which provides temporary stability to the plaque.

As described earlier, monocytes circulating in the blood migrate into tissues (particularly at the site of damage or injury) where they differentiate into macrophages [64]. Monocytes have high plasticity that enables them to adapt their phenotype when exposed to different stimuli [65]. There are two main classes of macrophages: classically activated type 1 (M1) and alternatively activated type 2 (M2). M1 macrophages are typically activated post injury or infection and tend to produce inflammatory cytokines, reactive oxygen and nitrogen species [66]. Conversely, M2 macrophages are induced by different stimuli from cytokines and toll like receptors to immune complexes [67]. M2 macrophages also predominately produce anti-inflammatory cytokines [68]. M1 macrophages tend to be the most common subtype of macrophages within inflamed atherosclerotic

plaques. Macrophage plasticity is also influenced by the presence of Oxidized LDL (oxLDL). OxLDL can accumulate in the vessel walls due to increased influx of LDL in the blood stream through permeable activated endothelium. Once the LDL permeates across endothelium, they undergo oxidation within the vessel wall. Some monocytes uptake oxLDL and undergo foam cell formation, which are commonly found in early atherosclerotic lesions [69].

State of the Art In Vitro Co-culture Methods to Study Atherosclerosis

In 1986, co-cultures of endothelial and smooth muscle cells were used by Weinberg et al to create a multilayered, tissue engineered blood vessel model where there were healthy, differentiated EC and SMC, which all exhibited appropriate physical and biosynthetic functions. Investigations carried out by Davies, et al. and Navab, et al. formed the basis of today's atherosclerosis models [70-72].

Studies that have been carried out to model atherosclerosis can be classified in two main categories: (1) indirect models, which contain two or more cell types without direct cell-cell contacts between cells of different types and (2) direct models, in which the multiple cell types coexist within the same volume. Within these categories there are two main subtypes of model systems: (1) static and (2) dynamic culture systems. Below, we explain the different variations of these models and culture systems in addition to how these models are utilized to study the different aspects of atherosclerosis *in vitro*.

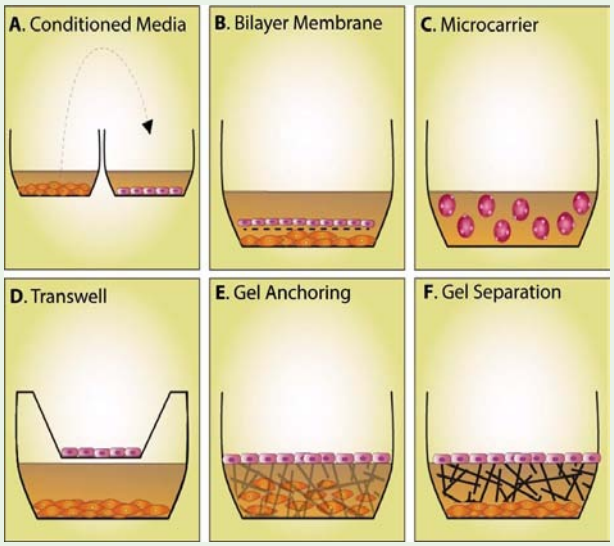

Figure 2: Current in vitro models of atherosclerosis. Models of atherosclerosis consist of combinations of: Endothelial Cells (EC), Smooth Muscle Cells (SMC) and monocytes or their respective byproducts, to study their cross talk and behavior during the initiation and progression of atherosclerosis in a mechanistic way. (A) Cells in question can be exposed to secretions of another cell type by means of transfer from a separate receptacle. (B) Bilayer and (C) microcarrier approaches both allow close proximity of two adherent cell types to the extent of allowing direct contact between them. (D) To decouple physical proximity but maintain immediate cell-cell signaling through secreted proteins the Transwell model has been used successfully. Some investigators also directly seed cells on top of each other to ensure cell-cell signaling while others create a biomimetic three-dimensional microenvironment using scaffolds of synthetic and native hydrogels either (E) for anchoring the cells spatially or (F) separating the cells.

Indirect Co-culture

Indirect co-culture enables different cell types to be grown in tandem without there being any direct cell-cell interaction. These types of studies are useful to investigate cellular responses between different cell types that make use of secretory pathways and cytokine production. The main variations of the indirect co-culture models are: microcarrier, scaffold, bilayer membrane, Conditioned Media (CM) and Transwell models.

Conditioned Media (CM): Spent media from cell culture contains soluble mediators that have been produced by the cells (Figure 2A). In this method of co-culture, the cell types involved are grown separately and the cell supernatant from one culture is introduced to another cell culture in order to elicit mediator-relayed cell-cell interactions. CM co-cultures enable experimental flexibility since it is possible to freeze the spent media and analyze it well after the experiment has been terminated. The CM model has been used in multiple different types of co-culture studies centered around atherosclerotic related events especially involving the use of SMC. Studies have been carried out using human monocytes and SMC in order to determine the interactions involved in SMC-mediated MMP production [73,74]. Vijayagopal, et al. used both human and bovine SMC and monocyte/ macrophage cultures to study the formation of lipid laden SMC [75]. SMC/macrophage co-cultures were incubated with LDL, Acetylated LDL (acLDL) and Lipoprotein Proteoglycan (PG) complexes obtained from human atherosclerotic lesions, for varying amounts of time. SMC were then isolated and their Cholesterol Ester (CE) levels were quantified. PG complexes that were exposed to SMC macrophage cultures resulted in the formation of aggregated complexes. SMC which were incubated with CM containing aggregated PG-complexes were found to stimulate cholesterol esterification in SMC. This was the same case as with the cells which were grown in SMC macrophage co-cultures but not when the different cell types were grown separately [75]. CM models have also been used to determine the effect of soluble mediators on SMC proliferation [76] and EC TF [77] expression when cells were grown in co-culture with macrophages and SMC respectively. The impact of monocytes on SMC calcification has been investigated. It was found that soluble factors produced by monocytes, namely TNF-α [78,79], enhanced calcification by increasing Alkaline Phosphatase (ALP) activity. Increasing the number monocytes, be it cell line (THP-1) or primary source (Peripheral Blood Mononuclear Cells, PBMC) increased matrix mineralization and ALP activity. ALP activity was not influenced by the addition of oxLDL to the cultures however bacterial Lipopolysaccharide (LPS) [78], Oncostatin M (OSM) and IFN γ [79] when used together resulted in increased ALP activity and *in vitro* calcification of SMC. Disadvantages of the CM model regard the use of the soluble mediators which may be ineffective in triggering cell response without cell-cell interaction. Additionally, these soluble factors may not be stable in media for a long period of time. Furthermore, when using CM models, the cell response has a uni-directional flow; therefore no feedback signaling is present between the cells types used which makes it an ineffective as a model representative of the cell behavior *in vitro*.

Microcarriers: The use of microcarriers enables some *in vivo* complexities to be modeled *in vitro* while simultaneously maintaining some of the properties of experimental cell culture (Figure 2B). Cells

can be grown in close proximity to one another. There is increased surface area between the cell types and easy separation of the cells. Davies, et al used microcarriers to create a co-culture between EC and SMC to determine the influence of the endothelium on LDL metabolism by SMC [70]. A calcium-dependent spheroidal co-culture of the two cell types was introduced where SMC organized them into the core of the spheroid with EC encapsulating the SMC core. This model resembles the *in vivo* angiogenesis of blood vessels. The co-culture of these two cell types resulted in a mature, quiescent EC phenotype. This was demonstrated by an increase in the number of junctional complexes within the EC monolayer, increase in the resistance of EC to apoptose as well as downregulation of PDGF-β expression [80].

Bilayer membranes: Bilayer membrane models, like microcarrier models, enable closer proximity between the cells used in the co-culture (Figure 2C). In this model, each cell type is seeded on one side of a porous membrane allowing cell-cell interactions at their basal side. Graham, et al. conducted studies on bilayer co-cultures of EC and SMC to better understand their *in vivo* interactions. Cells in these membrane systems could be kept in long term cultures, changes in cell-cell interaction up until confluency could be seen, and ease of separation of cells post intervention for the examination of the cells and ECM was observed [81]. Fillinger, et al. compared the co-culture of EC and SMC in a CM model with a bilayer membrane model, finding that, although in both types of models, the SMC demonstrated a similar morphology–spindle like shape with filamentous projections–within the bilayer model these projections transverse the membrane, resulting in contact between the two cell types. At day 14 of the culture, there was a greater effect on protein synthesis and SMC density. Therefore, it was found that bilayers are better models to study EC-SMC interactions than the conditioned media model [82]. Navab, et al. studied monocyte transmigration in EC-SMC co-cultures. It was ascertained that the endothelial layer was the main permeability barrier to the infiltration of monocytes and that monocyte transmigration through the endothelium is possible even without the use of a chemotaxis-inducing agent when EC and SMC were present in co-culture. However, the function of endothelium barrier was not lost upon the transmigration of monocytes [83]. Numerous studies were subsequently conducted in EC-SMC co-cultures to explore the adherence and chemotaxis of monocytes and leukocytes within the culture as well as their impact on the proliferative response of SMC. The presence of TNF-α led to the upregulation of ICAM-1 enabling greater monocyte adhesion and reduction of SMC proliferation, an effect that was mediated by the use of 5 mmol/ L of aspirin. Disadvantages of the bilayer membrane model are that (1) while ECs are found in a planar environment, SMC are usually found in 3D matrices that are not adequately imitated in the bilayer system, (2) the multi-stage seeding technique to coat both sides of the membrane with cells at separate intervals is inconvenient (3) cell interactions influenced by soluble mediators is unidirectional, as is the case with the CM models (4) overgrowth of cells on the pores of the membrane, will cause blockages and reduce or eliminate the cell-cell interactions [84].

Transwell: Transwell co-culture models enable the culture of two or more different cell types. In the Transwell (also known as a Boyden chamber) co-culture model, one cell type is grown on the lower chamber of the culture plate while the other is grown separately on the porous membrane filter insert (Figure 2D). Depending on the design of experiment, a third cell type, usually cells grown in suspension, or a drug candidate, or biochemical cues can be introduced on top of either the upper chamber or lower chamber. At its limit, a further attachment of a fourth cell type is also possible underneath the porous membrane, for a mixture of indirect and direct co-culture. To achieve co-culture, the lower chamber and filter insert can be combined. However, there is no direct cell-cell interaction between the cells within the Transwell insert and the lower chamber. This model type is useful to study cell-cell and cell-substrate interactions with regards to atherosclerosis. A majority of the studies carried out have examined SMC proliferation, calcification and apoptosis. Proudfoot, et al. and Fitzsimmons, et al. studied the influence monocytes and macrophages had on SMC proliferation [85] and procollagen secretion [86]. In this system, monocytes inhibited SMC growth and, while they do not cause procollagen degradation, monocytes do inhibit procollagen secretion by SMC. In co-cultures with EC, SMC and THP-1 cells, SMC proliferation was exacerbated by Advanced Glycation End Products (AGEs) [87]. Boyle, et al. studied SMC apoptosis, finding that macrophages triggered the production of TNF-α through autocrine pathways [88]. However, Cai, et al. showed that when monocytes bound to SMC, they continued to differentiate and apoptosis was stalled [89]. Studies demonstrated that SMC cultured with monocytes underwent calcification [90] and when monocytes developed lipid laden lysosomes, they could transfer these lysosomes into SMC if the two cell types were in direct contact with one another.

EC and monocyte interactions have also been studied in Transwell systems. Westhorpe, et al. exposed PBMCs to confluent layers of Human Umbilical Vein Endothelial Cells (HUVEC) grown to confluency within the upper chamber of the Transwell plate. Different forms of lipoproteins were located in the lower chamber. PBMCs transmigrated past the EC layer into the lower chamber which was activated using TNF-α either prior to or post transmigration. Monocytes were introduced to Transwell upper chambers without an endothelial layer to determine whether the cells would still transmigrate and differentiate. Monocytes transmigrated across the upper chamber regardless of the presence of lipids or endothelium. However, there was a substantial increase in the monocytes which formed foam cells when they were exposed to an activated endothelium [91].

The use of indirect co-culture lends itself to making simple models that are easy to control. Due to the ability to separate the cell types used, specific aspects and mechanisms of cellular behavior and interaction can be studied. The use of indirect co-cultures enables the creation of cellular environments specific to each cell type used within the model. However, because there is no direct cell-cell interaction between the cell types, the only intercellular communication occurs via paracrine signaling through soluble mediators. The disadvantages of using this model type are the necessity for cells to attach to plastic dishes or membrane filters (alone or within Transwell plates), forming non-physiological conditions, and short co-culture times that may result in less-than-adequate development of ECM interactions. Most importantly, the dimension of cellular behavior that is derived from the interaction between cells is not present, which is vital for mimicking and understanding the complexity of cellular mechanisms *in vivo* [92]. For this reason, it was paramount that direct co-culture systems be used to start modelling atherosclerosis *in vitro*.

Direct Co-culture

Direct co-culture involves two or more cell types of interest being overlaid upon one another or placed side by side indirect contact with one another. This technique is useful as it involves the three main types of cell interaction, which are signaling via cell-cell adhesion, cell-ECM adhesion and soluble factors.

Scaffold free: In order to study SMC apoptosis, Vasudevan, et al. plated SMC in serum-free media to which freshly isolated PBMCs were added at a 3:1 ratio. Cells were co-cultured for 48h with or without macrophage colony stimulating factor (M-CSF, a growth factor that induces survival, proliferation and differentiation of monocyte cells). Some co-cultures were pre-incubated with antibodies against ICAM-1. SMC apoptosis was contingent upon binding of monocytes to SMC, mediated by interactions between ICAM-1 and Mac-1 receptors [93]. Monocyte binding to SMC was exacerbated in diabetic conditions [94]. Chaterji, et al. created scaffold-free conditions to mimic both the healthy and diseased states of a blood vessel by altering the seeding densities of EC within EC-SMC co-cultures as well as adding soluble growth factors to induce desired cell phenotypes. The initial seeding densities for SMCs and ECs were $3\text{-}5\times10^4$ cells /cm^2 and $8\text{-}10\times10^4$ cells/cm^2, respectively. In an SMC monoculture, SMC differentiation markers, smooth muscle α-actin and calponin were upregulated by the addition of transforming growth factor-β1 (TGF-β1) and heparin. However, seeding near-confluent EC concentrations on SMC induced a higher expression of these SMC differentiation markers than the growth factors on their own. These expression levels were further increased when SMC were pre-treated with the soluble factors prior to seeding the EC layer. The soluble factors used were: 2.5 and 5 ng/mL transforming growth factor-β1 (TGF-β1), and 30 μg/mL heparin in a low-serum environment (1% [v/v] Fetal Bovine Serum [FBS]). Conversely, a hyperplastic state was induced when low concentrations of EC (3×10^4 cells/cm^2) were seeded onto the SMC. The study highlights the importance of the growth factors and EC seeding densities used when developing a co-culture model. Chaterji, et al. suggests that *in vitro*, in order to maintain the proper vascular tissue functionality, it is important to maintain the close proximity between the cell types that is found *in vivo*. Additionally the study shows that altering this structure is useful in mimicking a diseased state which in turn is invaluable in testing potential therapeutic molecules for treating cardiovascular diseases [95].

Scaffolds: Scaffolds are used in tissue engineering to create a three dimensional (3D) structure on which cells can be seeded and allow tissue formation. Appropriate scaffolds must possess characteristics that best enable cell growth, proliferation and expression of the desired cell phenotype: cell attachment to and migration into the scaffold, delivery of biochemical cues to and from the cells on the scaffold, biodegradability to ultimately enable the absorption and replacement of the scaffold with newly formed neotissue, diffusion of nutrients and cellular products into and out of the scaffold and creating a mechanical environment for the cells to stimulate cell bioconduction and bioinduction.

While different materials can be used to construct scaffolds, the two main categories most often used are biomaterials and synthetic materials. Biomaterials include substances like collagen, chitosan, alginate, gelatin, hyaluronan and fibrin [96]. The most common examples of synthetic materials used in tissue engineering scaffolds are Polylactic Acid (PLA), Polyglycolic Acid (PGA) and Polycaprolactone (PCL). The advantage of using natural polymers is that they are produced *in vivo* and can be sourced cheaply. Additionally, natural biomaterials have a greater degree of biocompatibility than synthetic materials since they have naturally occurring cell adhesion and binding sites [96]. Disadvantages include batch-to-batch variability, dearth of mechanical properties and the potential for contamination of the raw materials that could illicit immune responses in patients. Synthetic materials on the other hand have high reproducibility; can be produced in bulk and changes in mechanical properties such as degradation and composition can be modified easily. However, biocompatibility is limited due to the absence of cell adhesion and binding sites that are present in naturally occurring biomaterials and immune reactions can still be triggered in patients [96].

Scaffolds can also be two dimensional (2D) layers such as gels which are used to provide a coat on materials that would otherwise not be conducive to cell attachment and proliferation. Scaffolds used in atherosclerosis models have been used for multiple purposes: providing a 3D environment for co-culture of multiple cell types, highly reflective of the native microenvironment; anchorage of cells; creating a selection pressure for cells to express the required phenotype; allowing cells to grow within structures more accurately representing *in vivo* vessel geometry; and creating a 3D construct for cells to grow on to model the atherosclerotic vessel *in vitro*.

Gel Separation: VanBuul-Wortelboer, et al. grew SMC within a collagen lattice atop which EC were overlaid in order to understand the role EC play in modulating SMC behavior in co-culture (Figure 2E). In these conditions, SMCs adopted an elongated morphology as opposed to the polygonal shape that occurs when EC are cultured alone or on a Polystyrene (PS) substrate and EC additionally suppressed SMC proliferation [97]. Navab, et al. used an interesting approach to create a bilayer culture used to model monocyte transmigration in EC-SMC co-cultures. SMC were grown on gelatin layers and once sufficient autologously secreted ECM formed, ECs were laid on top [71,72]. LDL was introduced to these co-cultures, which brought about modification of the LDL and resulted in an upregulation of MCP-1. Exposure of monocytes to the co-cultures or CM from the co-cultures resulted in monocyte transmigration into the sub-endothelial space of the co-cultures. However, CM from individual cultures of EC or SMC did not induce monocyte migration. This effect was tempered by 91% with the addition of either antibodies against MCP-1, or HDL alongside LDL [72]. Further studies also showed that LDL, once pre-incubated with leumidins in an *in vivo* rabbit model resulted in modification of the lipoprotein into a stable complex which, once exposed to the co-cultures did not bring about modification of the LDL thus inducing monocyte transmigration [71].

Co-cultures were carried out by Takuku, et al. to study foam cell formation using rabbit aortic SMC and EC as well as human PBMCs (Figure 2F). MCP-1 was used to induce monocyte transmigration and subsequent differentiation into macrophages. Exposure to modified LDLs brought about foam cell formation [98]. Studies carried out by Wada, et al. corroborated these findings [99]. Phagocytic reverse transmigration across an endothelium, a common occurrence across vascular and/or lymphatic endothelia during atherosclerosis was investigated by Randolph, et al. HUVEC were grown on type I collagen gels upon which PBMCs, either unstimulated or stimulated with LPS, were placed as a control. Monocytes, but not lymphocytes

were able to migrate across the endothelial barrier. To test for reverse transmigration through the endothelium, PBMCs were incubated with the endothelial layer for a 1- or 2-h interval in order to facilitate migration of the monocytes into the sub-endothelial collagen. The apical surface of the endothelial layer was washed to remove any monocytes that had failed to transmigrate. Incubation was continued for 24h, and any monocytes that had reversed transmigrated to the apical side of the endothelial layer were washed off. The percentage of reverse transmigrated cells was ascertained by calculating the percentage decrease of monocytes beneath the endothelium at the end of the experiment compared to the number of monocytes present at the initial 2h interval. VIC7 antibodies against TF decreased this reverse transmigration by 77%. It was discovered that TF was not present on resting monocytes, but only on monocytes that had undergone the initial apical to basal transmigration across the endothelium. These findings indicate that monocyte adhesion to endothelium is enabled by the expression of TF on monocytes [100].

Lavender, et al. worked to optimize the conditions for the direct co-culture of EC on SMC and to assess the effect SMC had on EC function. The co-culture consisted of: culture substrate, basal adhesion proteins, a layer of porcine SMC, medial adhesion proteins and a layer of porcine EC. The optimal conditions for this model were: a polystyrene cell substrate, fibronectin basal protein, quiescent SMC with a subconfluent density, and a confluent density for EC. The use of fibronectin, laminin, collagen I and IV as medial adhesion proteins did not have a significant effect on EC adhesion. EC and SMC grew in two distinct layers and EC in co-culture formed tight junctions, however junction formation was not as developed as in EC monocultures. The co-culture was maintained for 10 days. It was demonstrated that EC could adhere to and be grown to confluence on a layer of quiescent SMC cells even when exposed to flow rates of 5 dyne/cm^2 for 7.5h. This study demonstrated that EC attached better on quiescent SMC compared to proliferative SMC [101].

Thus far, all the model systems that have been discussed, except the above by Lavender, et al. have been static models. Static co-cultures enable the stable growth of several cell types in tandem. While substantial discoveries have been made about atherosclerosis through static models as demonstrated by the large number of studies that has been detailed above, there are a significant number of limitations associated with these types of models. Static models neglect the flow and pulsatile conditions that are inherent in living, *in vivo* systems. One of the greatest setbacks of using static models for atherosclerosis co-cultures is the inability to account for the impact of shear stress on the different cell types involved. It has been mentioned before the role shear stress has on atherosclerotic regions and how the presence of shear stress influences the phenotypic switching in SMC or the alignment of EC is influenced by blood flow. In order to create more representative models, it was necessary to introduce dynamic parameters into atherosclerosis co-culture models.

Gel anchoring: Uses 3D hydrogels formed most often using either collagen or fibrin which have cells suspended within them. These gels recreate a 3D environment for cells, similar to *in vivo* conditions where the cells exist in more than one plane, typically within the ECM. Typically, to create a 3D gel, the desired cell type is placed in a gel solution that is left to polymerize. A second cell type can then be placed on top of the gel. A study carried out by Dorweiler, et al. used 3D gels within a static model to create multilayered SMC intima with formation of an ECM on which an endothelial layer was grown. The introduction of monocytes and lipids to this model led to monocyte transmigration and foam cell formation [102]. Subsequent studies have incorporated flow conditions into these models. Chen et al. studied the influence of both disturbed flow on WBC adhesion and transmigration in EC-SMC co-cultures [103], and the soluble mediator production induced by SMC and monocyte interactions [104]. Earlier studies showed the influence of flow and shear stress on the structure and functionality of porcine EC within EC-SMC co-cultures. EC grown in static on top of a porcine SMC layer were elongated and were oriented randomly. However, upon exposure to a shear stress ranging from (10-30 dyne/cm^2) within a parallel plate flow chamber, EC aligned them towards the direction of flow after 24 to 48h. This effect on the EC was maintained when they were culture on top of collagen 1 alone without the presence of SMC or flow conditions [105].

Cicha, et al. modelled the non-uniform shear stress profile at arterial bifurcations. HUVECs were seeded on slides and were exposed to 2.5 ng/mL of TNF-α at a flow rate of 9.6 mL/min (equivalent to 10 dyne/cm^2) for 2h. Adhesion of THP-1 monocytes was demonstrated using light microscopy. The combination of shear stress with the TNF-α treatment of the HUVEC led to a drastic increase in monocyte recruitment [106]. Li, et al. investigated vulnerable plaque destabilization using calcific nodules to model atherosclerotic plaques. Calcifying Vascular Cells (CVCs) were obtained from bovine ASMC cultures. These nodules were exposed to oxLDL and pulsatile shear stress (23 dyne/cm^2) for 2.5h in a pulsatile flow channel. The presence of oxLDL and monocytes increased the amount of CVC destabilization from the substrate as well as an increase in MMP activity, particularly MMP-9 [107].

Robert, et al. created a 3D tissue engineered artery was created under pulsatile flow conditions. The construct contained both HUVEC and human umbilical cord-derived myofibroblasts and was exposed to flow *in vitro* using a bioreactor. The histological analysis of the tissue demonstrated a basement membrane supporting a tight endothelium and multiple layers of smooth muscle cells. High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) were circulated through the construct. These lipoproteins migrated into the endothelium and were recovered both from within the EC as well as in the sub-endothelial intima. The endothelium was activated using TNF-α or LDL. Following that monocytes were introduced into the system and were found to adhere to the activated endothelium and subsequently transmigrate into the intima. This is the first example of a tissue engineered construct which simulates the *in vivo* physiological conditions [108].

Limitations of Current In Vitro Models

Compatibility between Studies

As discussed herein and summarized in Table 1, there have been many co-culture studies carried out to understand the nature of atherosclerotic mechanisms from monocyte attachment and infiltration of the endothelium to foam cell formation. These studies have been conducted using multiple cell sources: human, rabbit, bovine and porcine aortic cells being the most predominantly used. These studies have been carried out in a multitude of environments from 2D to 3D scaffolds, in gels and with or without the presence of

Table 1: *In vitro* co-culture methods to study atherosclerosis using Endothelial Cells (EC), Smooth Muscle Cells (SMC) and monocytes/ macrophages.

References	Source Cell type	Culture system	Focus	Key findings
[70]	Bovine AEC, ASMC	Static – Indirect - Microcarrier	Vascular EC-SMC metabolic interaction	LDL metabolism of SMC was influenced by the presence of endothelium
[115]	Bovine AEC, SMC	Static - Indirect - Scaffold	Construction of *in vitro* model of blood vessel	Could create multilayered vessel structure, EC and SMC were healthy and well differentiated, EC demonstrated both physical and biosynthetic functions
[97]	EC, SMC	Static - Direct	EC regulation of SMC proliferation in co-culture	EC suppress SMC proliferation
[83]	HAEC, HASMC, HBM	Static - Indirect - Bilayer	Monocyte transmigration into sub endothelial space	Monocyte transmigration occurred in EC-SMC co-culture even in the absence of chemotactic agent. Endothelium is primary permeability barrier to monocyte transmigration
[72]	HAEC, HASMC, HPMB	Static- Direct - Scaffold coated with gelatin	Monocyte transmigration	EC-SMC co-cultures when exposed to LDL and their conditioned media enable monocyte transmigration due to increased MCP-1 production. This effect can be counteracted with HDL and other anti-oxidants
[81]	EC, SMC	Static- Indirect- Bilayer	Bilayer model to study EC-SMC interactions *in vivo*	Model enables preservation of bilayer morphology, long term culture, and ease of separation of cells within culture post intervention
[71]	HAEC, HASMC, HPBMC	Static- Direct - Scaffold coated with gelatin	Monocyte transmigration	*In vivo* exposure of LDL to leumidins makes LDL resistant to modification when exposed to EC-SMC co-cultures. Modified LDL did not bring about monocyte transmigration.
[73]	Human saphenous vein SMC, PBMC	Static- Direct- 3D gel or Indirect - Conditioned media	Interactions between human SMC and monocytes causing MMP secretion	Exposure of SMC to monocytes and monocyte conditioned media resulted in secretion of MMP-1 and MMP-3 through the IL-1 dependent pathway
[105]	Porcine AEC, ASMC	Dynamic - Direct- 3D SMC gel, with EC monolayer on top	Regulation of EC structure and function by flow and shear stress within EC-SMC co-culture	EC align with direction of flow when exposed to shear stress. Presence of collagen allowed quiescent endothelium to be maintained in the absence of SMC or flow.
[75]	Bovine ASMC, AEC, P388D1, Human ASMC, AEC, Human monocytes	Static- Direct or Indirect- Conditioned Media	Formation of lipid laden SMC	Exposure of SMC-macrophage co-cultures tolipoprotein PG complexes (but not LDL or acLDL) resulted in CE synthesis and accumulation within SMC
[116]	Bovine ASMC, AEC	Static-Indirect- bilayer	EC influence on SMC phenotype in co-culture	EC influence SMC growth characteristics and phenotype by producing PAI-1
[117]	HAEC, HASMC, HPMBC	Static - Direct	Investigated role of HO in early atherosclerosis	HO-1 is induced by oxidized LDL which in turn reduces monocyte transmigration
[76]	HASMC, PBMC	Static - Indirect - Conditioned media	Effect of soluble macrophage mediators on the proliferation of SMC	SMC exposed to conditioned media from both activated and non-activated macrophages inhibited SMC proliferation
[82]	Bovine ASMC, AEC	Static-Indirect- bilayer and conditioned media	Comparison of conditioned media and bilayer EC-SMC co-culture models	Bilayer model more appropriate to study EC-SMC interactions due to physical proximity and interaction between cells
[100]	HUVEC, PBMC	Static - Direct - 2D gel	Reverse transmigration of phagocytes across endothelium	Adhesion of monocytes to endothelial cells is influenced by TF
[77]	HUVEC, HSMEC, HASMC, HUASMC, HUVSMC	Static- Indirect - Conditioned Media	SMC soluble factors on EC TF expression	Conditioned media from SMC demonstrating synthetic phenotype induced altered TF activity from established inducers of TF activity in EC.
[98]	Rabbit AEC, ASMC, Human PBM	Static - Direct - 2D gel	Foam cell formation	Within EC-SMC co-culture system, addition of monocytes was enhanced using MCP-1. Monocytes differentiated into macrophages. Exposure to modified LDL led to foam cell formation.
[85]	HASMC, PBMC	Static- Indirect - Transwell	Influence of monocytes/ macrophages on SMC growth	SMC growth was inhibited by monocytes
[86]	HASMC, PBMC	Static- Indirect - Transwell	Effect of monocytes on procollagen turnover from SMC	Monocytes inhibited procollagen secretion from SMC without affecting degrading procollagen
[74]	Human VSMC, THP-1 monocytes	Static - Direct or Indirect - Conditioned media/ Transwell	Investigating MMP-1 production from SMC - monocyte co-culture	Both direct co-culture and exposure of SMC to conditioned media resulted in MMP-1 formation. The former resulted in significantly higher MMP-1 production
[118]	Rabbit AEC, ASMC, Human PBMC	Static - Direct - 2D gel	Functional arterial wall model	Arterial wall constructed, HPM demonstrated transmigration and differentiation into macrophages which later formed foam cells within ECM upon exposure to modified LDL.
[119]	HUVEC, HUCSMC, PBL	Dynamic – Indirect- Bilayer - parallel flow plate	Patterns of leukocyte recruitment	Adhesion of lymphocytes was negligible both with EC and EC-SMC co-cultures. TNF-a treated co-cultures demonstrated greater leukocyte recruitment than EC monocultures

[120]	HCAEC, HCMSMC. Monocytes,CD4+ lymphocytes	Static – Indirect- Bilayer	Leukocyte adhesion, chemotaxis and SMC proliferative response	TNF-a up regulation of ICAM-1 on EC/SMC co-cultures studied. Adherence and chemotaxis by monocytes and lymphocytes reduced significantly with 5 mmol/L aspirin. Proliferative response of SMC after lymphocyte attack was also reduced significantly.
[79]	Human VSMC, THP-1, PBMC	Static - Direct or Indirect - Conditioned media/ Transwell	Calcifying phenotype in SMC	Both THP-1 and PBM cells induced ALP activity in SMC which resulted in the calcification of their extracellular matrix
[78]	Bovine CVCs, PBMC	Static - Direct or indirect - Conditioned media or Transwell	Monocyte influence on *in vitro* vascular calcification	Monocytes enhance vascular calcification both through direct co-culture and production of soluble factors
[121]	VSMC, Monocytes	Static - Direct	SMC apoptosis	Physiological concentrations of M-CSF induced SMC apoptosis
[122]	Human ASMC, PBMC	Static - Direct	SMC proliferation	Monocytes inhibit SMC proliferation
[93]	Human ASMC, Monocytes	Static - Direct	SMC apoptosis	Both M-CSF and monocyte induced SMC apoptosis requires Mac-1 and ICAM-1 mediated binding of monocyte to SMC
[88]	Human PBMCs, carotid, coronary medial, and aortic SMC	Static - Direct or Indirect - Transwell	Influence of TNF-a on SMC apoptosis	Macrophage induced SMC apoptosis is induced by TNF-a using both autocrine and direct pathways
[123]	hASMC, BAEC, BSMC	Dynamic - Scaffold	SMC migration through the vessel wall	PDGF stimulated hASMC migration into the stroma was halted by the induction of TIMP-1 production using TIMP-1 induced SMC
[89]	Human SMC, PBMC, THP-1	Static - Direct or Indirect - Transwell	Monocyte differentiation and apoptosis	Binding of monocytes to SMC increased their survival and differentiation
[124]	HUCEC, HUCSMC	Dynamic/ Static - Indirect- Bilayer	Effect of shear stress on EC-SMC interactions	In co-culture where only EC exposed to flow, SMC oriented perpendicularly to flow at higher shear rates and EC oriented themselves in the direction of flow. Without shear, EC gene expression of MCP-1 and oncogene-a brought about by co-culture with SMC was abated in the presence of shear stress.
[125]	Bovine AEC, SMC	Dynamic/ Static - Indirect	Effect of shear stress on EC-SMC lipoprotein uptake	Regardless of the presence of shear flow, LDL uptake in EC-SMC co-cultures was greater than in monolayers whereas Ac-LDL uptake remained unchanged. In the presence of shear flow, Ac-LDL uptake decreased for both co-cultures and cell monolayers.
[101]	Porcine EC, SMC	Static - Direct - 2D gel	Direct co-culture of EC on SMC and assessment of EC functionality	Confluent, adherent EC can be cultured on a layer of sub-confluent quiescent SMC
[126]	HUCEC, HUCASMC	Dynamic/ Static - Indirect- Bilayer	Inflammation related gene expression in EC due to co-culture with SMC	EC produce pro-inflammatory genes when cultured with SMC. This gene expression is inhibited when EC exposed to shear stress through inhibition of NF-κB activation brought about by SMC. Consequently, THP-1 adhesion was also curtailed.
[103]	PBL, CD14+ monocytes, PBMC, HUCSMC, HUCEC	Dynamic - Direct - 3D gel	Influence of EC-SMC on WBC adhesion and transmigration under disturbed flow	Co-culture significantly increases adhesion and transmigration of all WBC types. WBCs demonstrated different migration patterns.
[127]	HUVEC, HUCSMC	Dynamic - Indirect - Transwell, 2D gel plates/ capillaries	Platelet adhesion to EC within EC-SMC co-culture	Use of TNF-a and TGF-b with EC with a EC-SMC co-culture results in maximal levels of platelet adhesion due to the stimulation of vWF production in EC at low wall shear stress rates of 400 s-1.
[102]	HUASMC, HUVEC, Mono-Mac-6	Static - Direct - 3D gel	*In vitro* model of muscular artery to study early atherosclerotic events	*In vitro* model with multilayered SMC intima and endothelium with ECM was made. Addition of monocytes and LDL resulted in lipid insudation, monocyte transmigration and foam cell formation. IL-8 production was also demonstrated.
[128]	PMN, human EC, human SMC	Static - Direct	Sub endothelial infiltration and plaque destabilization	In EC-SMC co-culture, addition of LDL led to PMN adherence, transmigration and infiltration mediated by the IL-8 secretion pathway of SMC. This was followed by release of elastase and MMP-8 inducing EC apoptosis.
[104]	PBMC, PBL, CD14+ monocytes and HUCEC, HUCSMC	Dynamic - Direct - 3D gel	Influence of SMC-monocyte co-culture on soluble mediator production	SMC-monocyte co-cultures contribute to production of pro-inflammatory mediators
[94]	HASMC, THP-1, Mouse ASMC, WEHI78/24 monocyte	Static - Direct	Monocyte binding	Diabetes exacerbates the binding of monocytes to SMC
[129]	HAEC, HASMC	Static/ Dynamic (with pulsatile flow) - Direct/ Indirect (opposite sides of porous membrane)	Quiescent SMC influence on TNF a activated endothelial inflammatory response	EC cultured directly with SMC express fewer surface adhesion proteins after exposure to flow and TNF-a activation than EC cultured alone, or indirectly with SMC.

[95]	HAEC, ASMC, CSMC	Static-Direct	Altering cell concentrations and growth factors to model healthy and diseased blood vessels	Along with using the appropriate soluble factors, seeding near confluent concentrations of EC on SMC and sparse concentrations of EC on SMC can be used to model healthy and diseased states, respectively.
[130]	Human SMC, THP-1, mouse P388D1 macrophages	Static - Indirect - Transwell	Examined effects of Vitamin D activators on SMC matric calcification	SMC calcification was promoted by monocyte-SMC co-culture. This was inhibited by vitamin D receptor activators
[90]	P388D1 macrophages"	Static - Indirect - Transwell	Examined effects of Vitamin D activators on SMC matric calcification	SMC calcification was promoted by monocyte-SMC co-culture. This was inhibited by vitamin D receptor activators
[131]	Mouse VSMC, mouse bone marrow derived macrophage	Static - Indirect - Conditioned media	Macrophage plasticity	Switching of a macrophage phenotype towards M2 could be atheroprotective
[87]	HUVEC, HAVSMC, THP-1	Static- Indirect- Transwell plate	Influence of AGES on vascular cells	SMC proliferation induced by glycol AGES. Significant increase in cytokine expression levels within co-culture
[106]	HUVEC, THP-1	Dynamic - Direct - Microfluidic slides	Effect of non-uniform shear stress and TNF-a at bifurcations	Exposure of endothelium to shear stress and TNF-a caused increased monocyte recruitment
[91]	HUVEC and PBMCs	Static-Indirect - Transwell plate	Monocyte transmigration and foam cell formation	Monocytes form foam cells in the absence of lipids after transmigrating a TNF alpha activated endothelium
[107]	Bovine aortic smooth muscle cells, THP-1 monocytes	Dynamic - Direct culture - pulsatile flow channel	Characterizing calcific nodules to better understand vulnerable destabilization	Calcific nodules modelled atherosclerotic plaques. OxLDL and monocytes cause plaque destabilization through action of MMP
[108]	Human UCFMBs, HUVEC and monocytes from patients	Dynamic - Direct co-culture of cells on a 3D scaffold	Tissue engineered artery with 3D structure and pulsatile flow conditions to demonstrate atherosclerotic dysfunctions *in vitro*	Tight endothelium formation over basement membrane and multiple smooth muscle layers. Endothelium activation with LDL or TNF alpha resulting in monocyte adhesion and transmigration. LDL and HDL injected into the system and recovered within ECand sub-endothelial intima.
[132]	HASMC, PBM, U-937 monocyte like cell line	Static - Direct	Inflammatory mediator production from monocyte-SMC interaction in co-culture	Interaction between activated monocytes and SMC leads to resistin up-regulation in monocytes and ROS production in SMC. Resistin further exacerbates ROS production in SMC
[133]	HAEC, HASMC, PBM, Rat SMC	Static - Direct or Indirect - Conditioned media/ Transwell	Modulation of SMC behaviour by macrophages	Lipid laden lysosomes from macrophages are transferred into SMC *in vitro* when SMC-macrophages have direct contact
[134]	BAEC, BASMC, PCAEC, PCASMC, HUVEC, HUSMC, HAEC, HASMC	Dynamic - Direct co-culture of cells and indirect culture of cells	Wall shear stress on EC-matric, EC-EC and EC-SMC interactions	Wall shear stress disrupts endothelial monolayer. EC-SMC models more robust. EC-SMC interactions reduce EC-EC interactions and change EC phenotype and response to wall shear stress

flow. The issues that arise from having such a large variety of studies is that there are often conflicting results or a lack of reproducibility between different types of models. Frequently, there is a variation in the outcomes of direct and indirect model systems, typically with direct cell culture systems demonstrating the more amplified response. Particularly in the case of using cells from non-human species, the data obtained may not be representative of clinically relevant *in vivo* mechanisms of atherosclerosis. For example, the murine macrophage cell lines such as J774.A1 and RAW264.7 are missing apolipoprotein E expression genes, which are important in the development of atherosclerosis in humans [109]. This proves to be a problem when trying to use such models to gather clinically relevant data that can be used in patient treatment and drug development. Going forward it would be prudent to develop a standardized framework and cell type(s) to ensure compatibility between studies done by different labs worldwide.

Cell Source and Phenotype

Cells that are used to create atherosclerosis models are most often human primary cells. Primary cells are derived from living tissue and are meant to more closely represent the cell behavior *in vivo*. Primary cells are typically used at earlier passage numbers for a limited number of passages in order to ensure the *in vivo* phenotype is maintained. Unfortunately, primary cells are not widely available in large supply to carry out controlled studies; rather they are obtained from individual donors. These results in inherent variation in experimental results obtained. It will be challenging to identify a suitable, relevant patient cohort and a standard technique with which to obtain cell samples to be grown to a specific passage number and manipulated in order for them to express the required phenotype. Commercially purchased primary cells are the alternative and standardization of excision procedures provides some uniformity in the cells used for experiments. Cells behave differently *in vitro* as compared to their *in vivo* behavior within a native environment. Investigations by Potter et al. show that the glycocalyx-an important structure responsible for 'sensing' the hydrodynamic and mechanical environment at the EC surface, is missing from HUVEC and bovine AEC cultured in standard *in vitro* conditions [110]. The absence of the glycocalyx influences the surface chemistry of the EC which calls into question results obtained from studies that have previously been conducted on topics involving atherosclerosis, and endothelial barrier permeability [110]. Additionally, several different types of macrophages with different polarizations have been used in atherosclerotic models and these studies fail to acknowledge the influence on the model of having macrophages in either the pro-inflammatory M1 or anti-inflammatory M2 state. We too have shown that SMC from commercial source exhibit phenotypic changes through sequential passaging and prolonged culture of cells at a fixed passage number [111]. Thus, an additional challenge would be to optimize cell culture and conditions to suppress phenotype change, and further advocate fixed protocols.

In addition, building patient specific models is a reality considering the possibility of isolating endothelial progenitor and monocytic cells from patient blood. The former have to differentiate into EC and SMC [112,113].

Cell Substrate

When culturing cells *in vitro* for the purpose of cell expansion prior to actual experiments, cell culture wells, plates and flasks are used. Most often these culture materials are made of polystyrene or polycarbonate. Unlike the native microenvironment, these materials have high stiffness and they are not biomimetic of the ECM. Cell phenotype and behavior is greatly influenced by the stiffness of the cell substrate, particularly for cell proliferation and migration in SMC and cell morphology and tightness of endothelial barrier in EC. Our group has further shown that stiffness of the biomaterial can modulate SMC phenotype [114]. In order to create a more representative environment, cell culture material can be coated with biomaterials such as collagen or fibrin. When using biomaterials such as collagen or fibrin, there is inherent variation in the actual material as well as the composition of the gel solution and the resulting stiffness.

Clinical Applications and Future Trends to Improve Atherosclerosis Models

A majority of the studies that have been undertaken regarding atherosclerosis mechanisms have been carried out using animal studies and *in vitro* models. So far, the results of these studies have not been completely validated *in vivo* in humans. Thus, the results from these models cannot as yet be the predominant method used for the development of clinical strategies for disease treatment. Microfluidic devices have been devised for the high throughput testing of drug substances for the study of angiogenesis [33]. It is possible that further development of these techniques could lead to the creation of diagnostic devices for the study of diagnostic drug treatments for atherosclerosis. The major issue in the practical application of such models would be the long culture times required to create a well-developed construct that demonstrates the patients' *in vivo* blood vessel characteristics.

Concluding Remarks

Both direct and indirect co-culture models have been used to model atherosclerosis *in vitro*. Earlier studies were done in static culture systems, but in recent years the transition has been made towards modelling flow, pulsatile conditions and shear stress using dynamic modelling systems. The different phenotypes of the cells involved in atherosclerosis have also been described in great depth and have been correlated with cellular processes investigated within the models. Investigations have been carried out to gain better understanding of monocyte transmigration past the endothelium, differentiation and foam cell formation using monocyte and EC co-cultures. SMC and monocyte co-cultures were used to understand SMC apoptosis and calcification while EC and SMC cultures were utilized to understand the regulation of cell behavior and proliferation of each cell type by the other. The studies reviewed here provide insight into the relevance of *in vitro* models for the study of atherosclerosis, detailing the different ways atherosclerosis modelling is used for clinical applications. The various *in vitro* culture models illustrate the different mechanisms involved in disease progression as well as the importance of understanding cell-cell interactions involved. Overall, this review highlights the importance of *in vitro* modelling of atherosclerosis and the benefits these models will offer in gaining further understanding of the disease itself in addition to providing invaluable data on clinical screening of disease treatments.

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