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Tissue Engineering a Blood Vessel: Regulation of Vascular Biology by Mechanical Stresses

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Abstract Important to the tissue engineering of a substitute blood vessel is an understanding of those factors which regulate vascular biology. A major factor in this regulation is the mechanical environment imposed by the hemodynamics of the vascular system. In this the vascular endothelium plays a critical role, and over the past two decades much has been learned about the influence of hemodynamics on vascular endothelial biology, to a large degree using cell culture to study the effects of flow and cyclic stretch. In our laboratory, such studies are now being extended through the development of a model of the arterial wall involving the co-culture of endothelial cells and smooth muscle cells. The development of such a model and its use in the study of hemodynamic effects represents necessary steps in the evolution of approaches to tissue engineering a blood vessel. © 1994 Wiley-Liss, Inc.

Key words: tissue engineering, blood vessel, vascular biology, mechanical stresses, hemodynamic effects

The search for a satisfactory blood vessel substitute has been extended in recent years to include tissue engineering approaches. Tissue engineering in general is defined to mean the development of a biological substitute, i.e., one which utilizes living cells and/or other natural biological materials. For a substitute blood vessel this means the use of vascular cells and the extracellular matrix components indigenous to the vessel wall.

Early approaches to the tissue engineering of a substitute blood vessel focused on the use of a synthetic biomaterial seeded with a monolayer of endothelial cells (EC). For larger blood vessels synthetic vascular grafts had worked reasonably well; however, for small bore grafts, e.g., as needed in coronary bypass surgery, the degree of patency was totally inadequate. Through endothelial seeding it was thought that graft patency would be enhanced due to the natural endothelial interface provided between the flowing blood and the underlying synthetic graft material. In fact, some success has been achieved using this approach [1,2]. A different approach involves the co-culture of endothelial cells and smooth muscle cells (SMC), together with appropriate extracellular matrix (EMC) components. Efforts using this approach go back more than a decade [3–5], and this is the approach being taken in our laboratory. To date this approach, as promising as it is, still is in the stage of basic research and development.

As noted earlier, the tissue engineering of a substitute blood vessel involves the use of vascular cells and extracellular matrix components normally found in the arterial wall. Equally critical to this "mix" are the signals to which these cells will be exposed. These cellular signals, important to such processes as cell growth and differentiation, originate from three sources. First are those chemical signals which are derived from the fluid flowing through the vessel, which in vivo is blood. Next are those signals associated with the extracellular matrix. These ECM proteins are not just a biological "glue," which hold the vascular wall "mix" together; the extracellular matrix also provides biological signals which participate in regulating the biology of the vascular wall. Finally, there are the signals associated with the mechanical environment of the vessel wall, an environment which is imposed by the hemodynamics of the vascular system.

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In fact, these three different sources for cell signals are very much interactive. However, the one about which the least is known are those signals associated with the hemodynamically imposed mechanical environment. For this reason, our laboratory, as part of its long-term goal of tissue engineering a blood vessel, has chosen to investigate the influence of hemodynamics on vascular biology. The focus of this effort has been on the effects of flow and the associated shear stress on the biology of vascular endothelial cells; however, as discussed later, this effort is now being expanded to include vascular smooth muscle cells and the co-culture of such cells with endothelial cells.

HEMODYNAMICS AND THE VASCULAR ENDOTHELIUM

There is considerable in vivo evidence for a role of hemodynamics in regulating the vascular endothelium. To start with, the endothelium can be viewed as serving as a natural aerodynamic "tufting" of the arterial wall, with the orientation of the endothelial cells reflecting the direction of the flow in the immediate vicinity of the arterial wall [6,7]. Furthermore, changes in actin microfilament localization [8] have been observed with actin stress fibers aligned with the direction of flow in high shear regions, while in low shear regions the actin is mostly present in dense peripheral bands. It also has been shown that arterial diameter changes associated with alterations in blood flow are endothelium dependent [9] and that there is a shear stress regulation of arterial diameter both for normal and disease conditions [10,11]. In this the role of the endothelial cell may be as a shear stress sensor. Finally, using the pig model, studies have demonstrated that atherosclerotic lesion-prone regions, as identified by the incorporation of Evans blue dye, exhibit a thinner glycocalyx, enhanced macromolecule accumulation, increased monocyte recruitment, and increased endothelial cell turnover rates in comparison to the nonlesion prone, white or unstained regions [12,13]. The lesion prone regions were characterized by polygonally-shaped endothelial cells suggestive of a low shear environment, while the nonlesion prone regions were populated by highly elongated endothelial cells indicative of a high shear environment.

Fifteen years ago, laboratories began to turn their attention to the use of cell culture to study the influence of hemodynamic factors on vascular endothelial biology. These studies have involved the investigation of both the effect of flow [14–16], as well as that of cyclic stretch [17–19], where in this latter case the cyclic distension of the basement membrane in vivo by the pulsatile pressure is being simulated. To study this cyclic stretch effect, endothelial cells are grown on a compliant membrane which is then mechanically distended at a low frequency. Although studies using flow and cyclic stretch are in no way a complete simulation of the in vivo environment, the use of such cell culture systems does represent a situation where the response of vascular endothelial cells to a well defined mechanical environment can be studied. A variety of effects of both flow and cyclic stretch have been observed in cell culture. Included are changes in cell shape and orientation, cytoskeletal localization, and the mechanical stiffness of cells, cell proliferation, the synthesis and secretion of proteins, endocytosis, and intracellular signaling [20]. These effects of flow and cyclic stretch extend to the level of gene expression [21,22], with in some cases messenger RNA being upregulated, in others downregulated, and others yet there being no observed effect at all.

An important, unanswered question is how does an endothelial cell recognize the flow environment in which it resides, i.e., the signal(s) associated with its hemodynamically imposed mechanical environment? Having done so, how does it transduce this signal into the changes in structure and function already noted? There have been a number of studies of the mechanisms involved in this signal recognition and transduction [23–29]. These initially have focused on the latter, i.e., the second messengers associated with the transduction of a mechanical signal. What is now clear is that many of the second messengers known to be stimulated by flow-induced stresses are the same second messengers activated by chemical agonists. An even more important question is how does an endothelial cell recognize the mechanical environment in which it resides? There are many possibilities [30], and recognition quite possibly may be through multiple, parallel events. It is not even clear whether it takes place at the luminal surface and/or at the abluminal surface. It should be emphasized that just as there are different types of chemical agonists, each with their own effect, there are also different types of mechani-

cal environments, i.e., different forms of mechanical stimuli, each in effect being a different mechanical signal or "agonist."

ENDOTHELIAL CELL-SMOOTH MUSCLE CELL CO-CULTURE

If one is to understand vascular endothelial biology, it is not sufficient to understand simply the biology of endothelial cells, one must also understand the influence of the neighboring smooth muscle cells. For this reason a number of laboratories have developed techniques for co-culturing these two cell types.

EC and SMC can interact by two mechanisms, humoral and direct contact. In regard to the former, it has been shown that EC secrete both inhibitors and stimulants of SMC growth; however, when grown in co-culture with SMC, EC release more growth inhibitory activity [31]. One of these inhibitory substances was found to be a heparin-related glycoaminoglycan with a molecular weight of 10,000–15,000 [32]. Another important molecule due to its multipotency is transforming growth factor β (TGF-β) [33]. TGF-β was found to be the factor inhibiting EC growth when EC and SMC (or pericytes) were cultured in a culture system allowing direct contact between the two cell lines [34]. In a more recent study, Stewart et al. [35] showed that the release of endothelin, a potent vasoconstrictor peptide and a SMC mitogen, by EC is inhibited by co-culture with SMC. Another important molecule is PDGF which is known to stimulate different cell behavior in vitro and in vivo. All of this suggests that the co-cultures of EC and SMC developed up to now are only crude models of the vascular wall.

Along with the regulation of growth, cholesterol metabolism is also greatly affected by co-culturing EC and SMC. EC affect cholesteryl esterase (CE) activity in SMC by decreasing CE hydrolysis and low density lipoproteins (LDL) degradation and increasing CE synthesis and LDL uptake [36]. Alexander et al. [37], using transwell co-cultures, found an increased endothelial permeability to LDL in presence of SMC and a decreased LDL binding and uptake by SMC in presence of the EC. Incubating a co-culture of EC and SMC with LDL was observed to result in an induction of mRNA for monocyte chemotactic protein 1 (MCP-1), an increase in the level of MCP-1 in the supernatant, and an increase in monocyte transmigration through the co-culture [38]. Medium derived from co-cultures of human vascular cells was found to contain increased amounts of macrophage colony-stimulating factor (MCSF), TGF-β, fibronectin, and collagen and connexin 43 mRNA than do combined media from EC and SMC cultured separately [39]. These results imply that the co-culture of EC and SMC results in completely different endothelial properties, including alterations in its permeability to LDL and to monocytes.

Another important factor in a co-culture model is the presence of extracellular matrix proteins. The SMC and EC in vivo are surrounded by an intricate mixture of proteins such as collagens, elastin, laminin, fibronectin, and glycoaminoglycans. Several studies have shown that these proteins can affect the growth, differentiation and the cholesterol metabolism of both EC and SMC. Endothelial derived extracellular matrix can affect SMC growth, depending on its composition [40]. Collagen and fibronectin containing matrices promote SMC growth, whereas matrices containing heparan sulfate proteoglycans selectively inhibit identical smooth muscle cell populations [40]. ECM proteins can alter the response of SMC to LDL, e.g., collagen type I induces a decrease in SMC growth, but endothelial-derived ECM induces an increase in SMC growth following incubation with LDL [41].

Arterial SMC have very different characteristics when grown in culture than when they are found in normal blood vessels. When isolated and grown in culture, primary SMC change from a non-growing, contractile phenotype to a proliferating, protein secreting mode [42,43]. Factors influencing this change to a synthetic phenotype are the initial seeding density [42], the presence of extracellular matrix proteins [44], and the presence of endothelial cells [43]. However, subcultured SMC are always found in an irreversible synthetic state.

To study the influence of neighboring smooth muscle cells on EC growth in the presence of flow, we are developing a co-culture model of the arterial wall. In this co-culture model, porcine aortic SMC are seeded with soluble collagen I, and the collagen is allowed to polymerize in order to obtain a three-dimensional matrix with SMC. The cells bind to the collagen fibers and contract the gel to a final area that depends on many factors including the number of SMC seeded. Once the collagen lattice has fully contracted, porcine aortic EC are seeded at a very high cell density, with the cells rapidly becoming

confluent. Extracellular matrix proteins are sometimes layered on top of the gel, underneath the EC. Growth of arterial SMC in collagen gels has been studied before [45–47]. SMC grown in a collagen gel are much more elongated than cells grown on plastic and appear to have a phenotype close to contractile. Experiments have shown that the SMC grow very slowly in the gel as opposed to cells grown on a dish coated with collagen. EC grown on plastic have a cobblestone shape at confluence; however, when the cells are seeded on top of the SMC (with or without ECM), they are much more elongated, and a wave-like pattern of the endothelium is observed. The complete coverage of the EC and their elongation has been confirmed by scanning electron microscopy. No SMC was seen on top of the endothelium up to 10 days after EC seeding.

These initial studies demonstrate that it is possible to reconstitute a model of the vascular wall in cell culture in which the EC make a monolayer covering the surface of the gel and the SMC stay quiescent, not overgrowing the endothelium. The next step in the development of this model is the study of both steady and pulsatile flow conditions. Initial experiments indicate that, using this model and with exposure to flow at a shear stress of 30 dynes/cm^2 for 48 h, there was further EC elongation and also alignment. Further studies will be carried out, with the focus being on EC morphology and cytoskeletal structure and on both EC and SMC proliferation. Using this model, it will now be possible to more realistically investigate the influence of flow on vascular biology. This is necessary if the influence of the hemodynamically-imposed mechanical environment on vascular biology is to be understood, and this we believe is a prerequisite to the successful tissue engineering of a substitute blood vessel.

PROSPECTS FOR THE FUTURE

Once one better understands the role of hemodynamics in vascular biology, there still are other issues which need to be resolved before it will be possible to successfully tissue engineer a substitute blood vessel. These are listed in Table I, and of these, two important ones are the achievement of a smooth muscle cell density comparable to that which exists in vivo and the development of a vessel structure that will withstand the mechanical load imposed on a vessel by lumen pressure.

TABLE I. Issues That Need to Be Addressed in the Design of a Biological Substitute for Blood Vessel Replacement

EC properties for the substitute vessel (growth, thrombogenicity, vasoregulation)
SMC contractile phenotype
SMC density and growth (density on the order of 10^8 cells/cm^3 in blood vessels compared to 10^7 achieved in our co-culture)
Appropriate ECM composition (to obtain differentiated properties of the EC and SMC)
Tensile strength and elasticity of the substitute
Effect of mechanical stresses on the substitute blood vessel (shear stress, stretching, and pressure)

In our experiments to date, we have observed that the final smooth muscle cell density achieved within a collagen gel is directly related to the initial seeding density. However, it is a very non-linear effect; not only is cell number increased, but there also is greater gel contraction, thus a smaller volume. To date, the highest smooth muscle cell density achieved in our work is 10^7 cells/cm^3. This is an order of magnitude less than that found in vivo.

The second issue is that a substitute blood vessel must have the mechanical strength to withstand the load imposed by lumen pressure. In a normal artery this is provided by the vessel's elastin structure. This suggests that the use of elastin properly structured, perhaps synthetic elastin, might be a critical element in a tissue-engineered substitute blood vessel. The approach might involve the use of a biomaterial to provide a three-dimensional structure for growing the smooth muscle cell layers. As part of addressing the vessel's mechanical properties, it will be important to examine the effect of any compliance mismatch between the substitute vessel and the existing native vessels.

Although these two issues, as well as others not discussed, will not be easily solved, the goal of tissue-engineering a blood vessel is an important one. Taking coronary artery bypass surgery (CABS), as an example, 400,000 CABS procedures were performed in the U.S. in 1990, and this figure has more than doubled since 1982 (48). The death rate after CABS over 18 years is 33% and the graft patency rate is 50–70% after 10 years with an observed acceleration of graft closure after 5 years [49]. Moreover, a significant percentage of the U.S. population does not have acceptable native vessels available. In addi-

tion, with the high rate of graft closure over 10 years, other individuals have had previous bypass surgery and thus also do not have native vessels available. Finally, there are other forms of vascular surgery where a tissue-engineered small-bore vascular graft of high patency would have considerable use. Thus, in spite of the problems still remaining to be solved, the goal remains an important one.

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