

Learning from vascular remodelling

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Summary

The positioning of a hollow silicone collar around the carotid artery of a rabbit induces many changes of early atherosclerosis including intimal proliferation of smooth muscle cells. This occurs below an intact endothelium indicating that endothelial damage is not necessary for smooth muscle cell proliferation. The endothelium may in fact produce substances that control processes occurring in the intima. Vascular endothelial growth factor (VEGF) is an angiogenic agent that is produced by cultured vascular smooth muscle cells. The combination of hypoxia and factors such as platelet-derived growth factor, tumour necrosis factor alpha, basic fibroblast growth factor, and interleukin-1 β lead to synergistic production of VEGF by cultured smooth muscle cells. VEGF receptors are present predominantly on the endothelium and may be an important target for modulating the response to damage, hypoxia and inflammation. Transfection of the gene for VEGF resulted in inhibition or regression of intimal hyperplasia induced by the silicone collar in the rabbit. Studies suggest that the two mediators responsible for this inhibition of smooth muscle cell proliferation are nitric oxide and prostacyclin, which are produced by cultured endothelial cells incubated with VEGF. Thus, VEGF produced by smooth muscle cells in response to hypoxia, damage or inflammation, acts on specific endothelial receptors to produce nitric oxide and prostacyclin, which inhibit smooth muscle cell proliferation. Failure of this process could give rise to intimal hyperplasia. Early clinical studies of VEGF transfection from the outside of human arteries using a biodegradable collar are in progress.

Keywords: vascular smooth muscle, proliferation, vascular endothelial growth factor, nitric oxide, prostacyclin, atherosclerosis, hyperplasia

Introduction

Rokitansky and Virchow stated that atherosclerosis began on the inside of blood vessels. However, it is possible that the adventitia might have an influence over the intima of the endothelium. Many years ago, we described a model whereby we put a hollow silicone collar around the outside of the carotid artery of a cholesterol-fed rabbit [1]. Substances could be placed into the void of the collar to see if they were pro-atherosclerotic. Interestingly, if the hollow space of the collar was left empty or filled with saline, there were significant histopathological changes in the intima between the internal elastic lamina and the endothelium compared with the contralateral carotid artery without a collar. Many of the changes were similar to those seen in

early human atherosclerosis: foam cell formation (which is macrophage-derived) and smooth muscle cell infiltration. This was purely a local effect because there was no change on the contralateral side. If rabbits were fed a no-cholesterol diet, there was only smooth muscle cell infiltration into the intima as identified using a monoclonal antibody to smooth muscle cells [2]. The smooth muscle cells aligned in the normal direction in the media. This is exactly what is found in intimal proliferation of smooth muscle cells following re-stenosis after angioplasty. The smooth muscle cells seem to align in the direction of the flow of blood.

The role of macrophages

In rabbits fed a no-cholesterol diet, there are no macrophages in the intimal lesion, whereas rabbits fed on a very low cholesterol diet for 7 days have macrophages that

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become foam cells in the intima. Therefore, this is a useful model for dissecting out the role of smooth muscle cells in hyperplasia and in restenosis after angioplasty, and the role of macrophages.

Interestingly, low power scanning electron microscopy demonstrated that the lesion in the intima occurred below a completely anatomically intact endothelium. However, it is widely believed that endothelial damage causes atherosclerotic lesions. Thus, we have learnt that endothelial damage is not necessary for the development of lesions that look like early atherosclerosis (at least in the rabbit). If you use a model that destroys endothelium in order to produce smooth muscle cell proliferation then, by definition, you are going to miss all those changes that might occur in the endothelium and that might be important in controlling what happens in the intima.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) might be involved in some of these changes in the vessel wall. It is a powerful protein that may be involved in a variety of processes. To date, it has been described as an angiogenic molecule that is needed in the fetus for blood vessel growth. Even the heterozygous knockout is lethal after a few days of embryonic life. VEGF has established biological functions *in vivo*: it causes angiogenesis in tumours and in the embryo. In addition, it has been shown to cause endothelial cell proliferation *in vitro*. There is one VEGF gene with several isoforms, but there are now several forms of VEGF (A, B, C, D and E have been described). VEGF acts at the tyrosine kinase receptors KDR/Flk-1 and Flt-4.

It is known that hypoxia causes VEGF production in the fetus [3]. When the limb bud of the fetus grows it becomes hypoxic at the end. The message given to the vessel supplying that limb bud is VEGF, which gives the signal to cause division and proliferation of endothelial cells.

To determine whether smooth muscle cells could produce VEGF in response to a hypoxic stimulus, we incubated vascular smooth muscle cells from rat, rabbit or human aorta in a culture medium where we could control the amount of oxygen. Levels of VEGF mRNA were analysed by Northern blot hybridization. As the oxygen in the culture medium was decreased, there was an increase in mRNA for VEGF [4]. Even more interestingly, vascular smooth muscle cells incubated with platelet-derived growth factor (PDGF) at normoxia produced VEGF. Furthermore, with a combination of PDGF and hypoxia, there was a synergistic increase in the amount of VEGF produced by smooth muscle cells [5]. Similar results have been obtained with basic fibroblast growth factor (bFGF) [4], and also with interleukin (IL)-1 β and tumour necrosis factor (TNF) α [6].

The results of these studies suggest that VEGF is a

messenger that travels from the smooth muscle cell to the endothelium, which has receptors for VEGF, i.e. KDR/Flt-1 and Flt-4. KDR receptors have been frequently reported on endothelium but appear to be absent from monocytes [7]. Therefore, VEGF receptors may be important as a drug target because they will give specific effects. Could it be that VEGF is produced in smooth muscle cells in response to damage (bFGF), hypoxia, or inflammation (TNF α and IL-1 β)? Traditionally, it is thought that the endothelium controls the function of the vessel wall. Could it be that the vessel wall also controls the function of the endothelium?

The role of VEGF in the vessel wall

To look at the role of VEGF in the vessel wall, we studied the effect of VEGF gene transfer on smooth muscle cell intimal hyperplasia produced in rabbits with a silicone collar around the carotid artery [8]. In the reservoir of the collar the gene for VEGF in a plasmid was delivered with a vehicle such as a liposome or an adenovirus or retrovirus. This resulted in the transfection of the gene from the outside of the vessel wall with about 50% efficiency. Transfection of a control gene with no function resulted in intimal hyperplasia, whereas transfection of the gene for VEGF completely abolished intimal hyperplasia [8]. Not only could VEGF inhibit intimal hyperplasia, it also could cause regression of existing intimal hyperplasia.

What could be happening to the endothelium that could cause inhibition of smooth muscle cell intimal hyperplasia? There were two candidates: nitric oxide (NO) and prostacyclin (PGI₂). When cultured endothelial cells were incubated with VEGF at physiological concentrations, nitrite (a metabolite of nitric oxide) was produced rapidly (Fig. 1). In addition, VEGF caused prostacyclin release from cultured endothelial cells as seen by measuring its metabolite 6ketoPGF_{1 α} [9].

Therefore, the hypothesis is that VEGF is produced from smooth muscle cells and acts on specific receptors on the

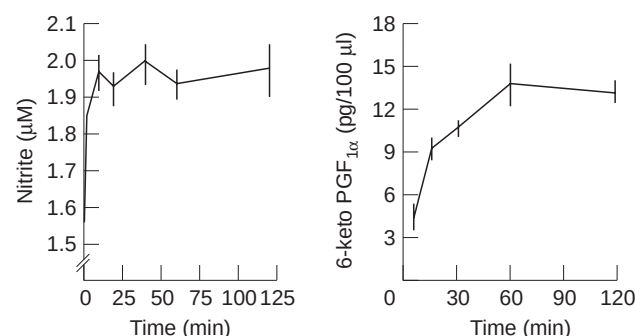


Fig. 1. Effect of VEGF on nitrite and PGI₂ production (measured as the 6ketoPGF_{1 α} metabolite) by cultured endothelial cells.

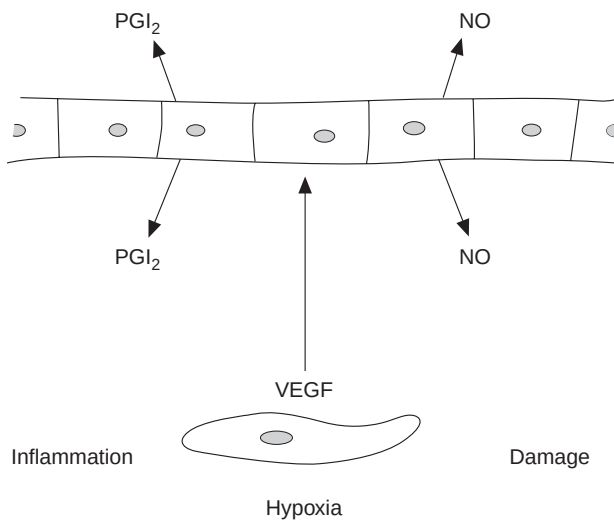


Fig. 2. Model of VEGF production by smooth muscle cells that produces NO and PGI₂ to inhibit smooth muscle cell division following exposure to 'damaging' stimuli.

endothelium to produce nitric oxide and prostacyclin, which inhibit smooth muscle cell division that takes place after damage, hypoxia or inflammation of the vessel wall (Fig. 2). In other words, these two agents may damp down the repair response in the vessel wall. Most of the stenosis that occurs in arteries is an inappropriate repair to damage. It is known that prostacyclin and nitric oxide work synergistically through cyclic AMP and cyclic GMP, respectively, to inhibit smooth muscle cell replication [10]. Moreover, nitric oxide and prostacyclin inhibit platelet aggregation, and together they produce a synergistic inhibition of platelet aggregation. Could it be that these two agents have a fundamental cytoprotective effect and that the failure of this process gives rise to intimal hyperplasia?

Administration of a nitric oxide synthase inhibitor L-NAME to rabbits with the silicone collar around their carotid arteries, reduced the inhibitory effect of VEGF on smooth muscle cell proliferation by 85%. This indicates that nitric oxide can modulate this effect of VEGF on the smooth muscle cells *in vivo*. Currently, we are performing similar experiments with indomethacin and prostacyclin and are investigating the effects of VEGF on platelet aggregation.

A phase I study is about to begin in Finland whereby the gene for VEGF will be transfected from outside human arteries to demonstrate that this occurs safely. Subsequently, a phase II study of the inhibition of intimal hyperplasia will be performed using a biodegradable collagen collar to deliver the gene for VEGF.

Conclusions

The work described above introduces several concepts. Firstly, that the walls of structures might be controlled by

the cells which line them. It is possible that just as the arterial wall might be controlled by the endothelium, the epithelium might in some way control events within the airway wall. Secondly, if damage to the endothelium had been produced in the rabbit model, we would never have found that the endothelium plays a role in producing substances to control smooth muscle cells. Gene therapy for the cardiovascular system has been unsuccessful so far for two main reasons. Firstly, putting the gene into the blood allows it to circulate throughout the body causing toxicological problems. Secondly, it has been difficult to obtain high enough concentrations of the gene. The biodegradable collar will probably overcome these problems and confine the gene locally. Indeed, when a mouse gene is inserted into a rabbit in this way, the mouse gene stays in the arterial wall and there is no mouse protein circulating. Additionally, the concentration of the gene in the reservoir can be increased until the required concentration in the vessel wall is reached.

Discussion

Chung: One of the things that is quite different is that vascular smooth muscle is much more responsive to proliferation than airway smooth muscle. The changes you get in your model are very difficult to get in the airways of an animal model. We have to expose a sensitized animal to repeated allergen exposure and the degree of proliferation is very small. Secondly, I learned that vascular smooth muscle is very sensitive to hypoxic stimuli. I don't know if airway smooth muscle does that; it may be possible.

Martin: I believe that the collar is causing hypoxia in the outer wall by occluding the vasa vasorum. We see induction of HIF1 α within one day followed by VEGF about 6 h later. So there is a hypoxic sensing system in the smooth muscle cells and they react very rapidly. I don't know if anyone has compared vascular smooth muscle cells with airway smooth muscle cells.

Hirst: We have grown them together in the same incubator but we haven't necessarily done the same experiments on them. We have looked at hyperoxia in neonatal human tissue, which would be relatively hypoxic *in utero*, and exposed it to increasing concentrations of oxygen. In the presence of a growth factor, but not in the absence of a growth factor (whether that be PDGF, alphathrombin, FGF or serum), exposure from 2 to 3 kPa to 50 kPa of oxygen and peaking at about 10–12 kPa oxygen, there is proliferation of neonatal human airway smooth muscle. We did the same experiment with the epithelium from these preparations and added the condition supernatant to the smooth muscle cultured from the same tissue source. This also induced proliferation.

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