Transforming Growth Factor β and Atherosclerosis: So Far, So Good for the Protective Cytokine Hypothesis

David J. Grainger

Abstract—The role of the anti-inflammatory cytokine transforming growth factor β (TGF-β) in atherosclerosis has been the subject of considerable debate for a decade. In the early 1990s, we postulated that TGF-β played an important role in maintaining normal vessel wall structure and that loss of this protective effect contributed to the development of atherosclerosis. We termed this the protective cytokine hypothesis. This proposal was slow to gain broad acceptance, however, because at that time there were little data available on the role of TGF-β during the development of atherosclerosis but much information about its role during trauma-induced neointima formation. Because TGF-β apparently aggravates neointima formation, both by inhibiting endothelial regeneration and by promoting fibrosis, it was difficult to accept that its presence might ameliorate the superficially similar atherogenesis process. But several recent studies revealed beyond doubt the fact that TGF-β protects against lipid lesion formation, at least in mouse models of atherosclerosis. Therefore, two important questions remain. First, is the role of TGF-β in vascular biology similar in humans and in mice? Secondly, how important, compared with defects in thrombosis or lipoprotein metabolism, is the protective role of TGF-β during atherogenesis? (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: TGF-β ■ myocardial infarction ■ inflammation

The transforming growth factor β (TGF-β) superfamily consists of a large number of structurally related cytokines that participate in a vast range of biological processes from axis specification and tissue differentiation in early development through to regulation of a range of immune factors, that TGF-β1 results in perinatal death after unbridled leukocyte extravasation in almost every organ system. Because accumulation of leukocytes, particularly macrophages but also T cells, is a characteristic signature of the change from normal vessel wall structure to early atherosclerotic lesion, we postulated that lowered TGF-β activity in the vessel wall might lead to leukocyte accumulation, in
parallel with increased VSMC proliferation, migration, and dedifferentiation.\textsuperscript{3}

Over a period of years, a range of other potentially protective effects of TGF-\(\beta\) in cell culture were identified, including suppression of proinflammatory adhesion molecule expression by the vascular endothelium,\textsuperscript{15} and reduced foam cell formation in cultured macrophages.\textsuperscript{16} Recently, Mallat and Tedgui\textsuperscript{17} have provided an excellent overview of the immunomodulatory role of TGF-\(\beta\) during the development of atherosclerosis, in which they highlight the central role of TGF-\(\beta\) in the regulation of T-cell biology and its implications for the chronic inflammatory component of atherogenesis. Recent evidence from Gojova et al\textsuperscript{18} underlines the role of TGF-\(\beta\) in regulating this process.

The role in regulating this process is likely to reflect the complexity of the role of TGF-\(\beta\), which signals specifically in the T-cell population, whereas generalized suppression of TGF-\(\beta\) levels (using neutralizing antibodies,\textsuperscript{19} soluble receptor fragments,\textsuperscript{20} or heterozygous deletion of the \textit{tgfb1} gene\textsuperscript{21}) led to either no change in lesion size or an increase in lesion size. This is likely to reflect the complexity of the role of TGF-\(\beta\) in the vessel wall, with important effects not only on the leukocyte populations but also on the endothelial and vascular smooth muscle cell compartments. This review therefore complements the article by Mallat and Tedgui\textsuperscript{17} by focusing primarily on the impact of TGF-\(\beta\) signaling on the vascular smooth muscle cell population in the vessel wall.

This interplay between the leukocyte and smooth muscle cell compartments is likely to be particularly important when considering the impact of TGF-\(\beta\) signaling on ECM production. Elaboration of ECM is one of the major functions of the smooth muscle cell, and the balance between leukocytes and smooth muscle cell populations in the developing atherosclerotic plaque is therefore a major determinant of plaque stability.\textsuperscript{22} Consequently, TGF-\(\beta\) is likely to play a central role in regulating this “stability balance” in at least two distinct ways: (1) through its immunoregulatory role it suppresses leukocyte recruitment; and (2) it is one of the most powerful fibrogenic cytokines described to date. TGF-\(\beta\) therefore not only protects against the initial formation of fatty streak lesions by maintaining normal blood vessel wall structure\textsuperscript{19,21} but also protects against the development of unstable lesions by driving matrix production by the smooth muscle cells of the fibrous cap.\textsuperscript{19,20}

Potentially, suppression of TGF-\(\beta\) signaling is progressively and doubly dangerous. To lose TGF-\(\beta\) activity temporarily is merely careless (leading to formation of stable matrix-rich lesions), but to lose TGF-\(\beta\) activity repeatedly is potentially fatal (leading to formation of leukocyte-rich, matrix-poor lesions that are at high risk for rupture).

More recently, a plausible molecular basis for this double role of TGF-\(\beta\) during atherogenesis has evolved: McCaffrey et al demonstrated that VSMCs (the cells that make all the ECM in the vessel wall) had different TGF-\(\beta\) receptor expression profiles in atherosclerotic lesions compared with normal vessel wall.\textsuperscript{23} In the diseased vessel, the cells dominantly expressed the type I TGF-\(\beta\) receptor, whereas in normal vessel wall the type II receptors dominated. This change had functional consequences, at least in vitro. The type I dominated VSMCs responded to TGF-\(\beta\) very differently from the type II dominated cells, by massively elevating ECM production.\textsuperscript{23} In contrast, the type II dominated cells barely increase ECM production at all in response to TGF-\(\beta\). We confirmed and extended these studies, demonstrating that when VSMCs from healthy artery are first dispersed into cell culture, they have high levels of type II receptor and respond to TGF-\(\beta\) by increasing contractile protein expression, but not ECM production. In the absence of added TGF-\(\beta\), over a number of cell cycles in culture, the ratio of type II to type I receptor decreases, and the cells switch their responses over. Now TGF-\(\beta\) stimulates ECM production but not contractile protein expression. Most interestingly, if the freshly dispersed cells were maintained continuously in TGF-\(\beta\), they maintained high levels of type II receptor indefinitely in culture, and with it maintained a differentiated phenotype with relatively low ECM production.

Taken together, these studies allow a plausible molecular model for the role of TGF-\(\beta\) in the vessel wall to be constructed (Figure 1). High levels of TGF-\(\beta\) maintain a...
feedback loop in which type II receptor remains high and the vessel wall retains its differentiated phenotype with lots of contractile proteins and relatively little ECM. If some external factor intervenes to reduce TGF-β activity sufficiently in a particular region, the VSMCs respond not only by dedifferentiating, proliferating, and migrating into the vessel intima but also by reducing type II receptor expression. This is the “first hit” of reduced TGF-β activity that promotes early fatty streak lesion formation.

However, the changes in TGF-β receptor expression patterns have the effect of locking in the vessel wall changes that have occurred, because even if TGF-β activity returns to normal, those dedifferentiated intimal VSMCs respond by producing ECM rather than by returning to the contractile state. The result would be a stable matrix-rich atherosclerotic plaque. However, if TGF-β activity remained suppressed or was lowered still further, then the “second hit” of reduced TGF-β activity occurs. Enhanced recruitment of leukocytes to the plaque coupled with reduced ECM production by the remaining VSMCs results in formation of a rupture-prone plaque typical of those that go on to have clinical sequelae, including myocardial infarction and death.

Such a model provides one possible explanation for a range of features of the atherogenic process, but is it correct? And what might trigger localized suppression of TGF-β activity?

**Direct Evidence Supports the Protective Cytokine Hypothesis in Mice**

Genetic manipulation has become the standard method for determining the importance of gene products in disease processes. The gene of interest is knocked out, and the impact on disease progression monitored. Unfortunately, the *tgfb1* null mouse dies soon after birth from severe multifocal inflammation, preventing us from studying vessel wall architecture in the absence of TGF-β. Two approaches have been taken to get around this problem: *tgfb1* null mice can be bred on a SCID background, allowing them to survive into adulthood; alternatively, mice heterozygous for the *tgfb1* deletion are studied. These *tgfb1*−/− mice have lower levels of TGF-β1 in a number of tissues, including the blood vessel wall, and provide a direct method to study the impact of lowered TGF-β1 levels on vessel wall architecture.

Adult *tgfb1*−/− mice have reduced levels of smooth muscle differentiation markers, including smooth muscle specific-α-actin and smooth muscle-specific myosin heavy chain in the aortic wall, consistent with the expected effect of reduced levels of TGF-β1. However, neither *tgfb1*−/+ mice on a SCID background nor *tgfb1*+ mice showed signs of endothelial activation (marked by VCAM-1 and ICAM-1 expression). Interestingly, when the mice were fed a high-fat diet, endothelial activation and lipid deposition was observed in the *tgfb1*−/− mice but not in their wild-type littermate control. These observations demonstrate that reduced TGF-β1 production renders the vessel wall susceptible to endothelial activation and development of early stage lipid lesions when subjected to a proatherogenic challenge. This study, however, provides no information as to when or where the level of TGF-β1 must be reduced to promote susceptibility to atherogenesis.

More recently, several other groups have used different methodological approaches to draw similar conclusions. Both Mallat et al and Lutgens et al used in vivo neutralization approaches to show that depletion of TGF-β1 in the blood vessel wall of adult apoE-deficient mice was sufficient to exacerbate lipid lesion development. The amount of lipid deposited was increased in both studies, as was the number of macrophages and other inflammatory cells accumulating in the developing lesion. Lutgens et al went on to demonstrate that neutralizing TGF-β decreased ECM deposition, confirming the role of this cytokine in regulating the balance between an unstable, proinflammatory lesion phenotype and a stable, matrix-rich phenotype.

Taken together, these studies provide strong evidence that TGF-β protects against unstable plaque development in at least two ways. TGF-β acts constitutively to suppress the changes in vessel wall architecture that are associated with lipid lesion development in mice (the “first hit” of reduced TGF-β). Decreased TGF-β activity later during the atherogenic process results in a switch to an unstable inflammatory lesion phenotype (the “second hit” of reduced TGF-β).

**What Factors Might Trigger Suppression of TGF-β Activity in Humans?**

One of the best understood local triggers for suppression of TGF-β activity in the blood vessel wall is the variant lipoprotein particle Lp(a). Lp(a) consists of an additional protein component, apo(a), covalently bonded to the apoB of an LDL particle. The distinguishing apo(a) protein has sequence homology to plasminogen but has been inactivated in the catalytic triad, rendering it incapable of yielding an enzymatically active form equivalent to plasmin. Rifkin et al first demonstrated that Lp(a) could inhibit TGF-β activation in cell culture, an observation that has been repeated many times. We extended these studies and demonstrated that transgenic expression of apo(a) in mice, which have no equivalent endogenous gene, results in impaired TGF-β activation in the blood vessel wall. In a further study, we demonstrated that apo(a) accumulates preferentially at sites where TGF-β activation is already suppressed, leading to a positive feedback loop that explains the highly focal suppression of TGF-β activity in apo(a) and Lp(a) transgenic mice. Feedback loops such as these may contribute to the focal nature of atherosclerotic lesions, which affect some regions of the vasculature but not others within the same individual. To date, however, there are no data to determine whether the presence of Lp(a) lowers TGF-β activation in humans.

A range of other factors have also been postulated to regulate TGF-β activation (Figure 2), including proteases that are thought to cleave the LAP-promoting release of mature TGF-β, binding proteins that alter the conformation of the complex promoting its dissociation, and physicochemical factors (such as acidity and radiation) that also favor dissociation of protein complexes. Recently, Rifkin et al have proposed a unifying scheme, suggesting that the LAP dimer acts as a “sensor” component of the latent TGF-β complex,
integrating the activity of multiple environmental factors to tightly regulate the release of the “effector” TGF-β complex. Thus, factors such as PAI-1 that are known to be elevated during atherogenesis,34 or urokinase and plasmin activity (which are known to be suppressed34), are likely to contribute to local suppression of TGF-β activity at the site of vascular lipid lesion formation.

Various mechanisms also link defects in lipid metabolism to suppression of TGF-β activity. After a standardized high-fat meal, TGF-β activation is transiently suppressed. Several mechanisms may contribute to this. PAI-1 production is stimulated by dietary fat load, to an extent that depends on the 4G/5G genotype in the PAI-1 promoter.35 Suppression of TGF-β activation was similarly associated with the PAI-1 promoter genotype, suggesting that PAI-1 may contribute to the reduction in TGF-β activity. Additionally, the hydrophobic TGF-β protein can be sequestered into triglyceride-rich lipoprotein particles, reducing its ability to interact with its signaling receptors,36 which may exacerbate the reduction in TGF-β activity seen after dietary fat loading.

TGF-β activity may also be suppressed at regions of low shear stress (for example, at vessel branch points37). These preliminary observations in human studies have been replicated in studies of the rodent vasculature.26 Again, several molecular mechanisms may contribute, including a direct shear response element in the tgfβ1 gene promoter38 and a flow-dependency in the expression pattern of the inhibitory Smad proteins, Smad6 and Smad7.39

As in mice, the definitive experiment to implicate TGF-β in the atherogenesis process in humans would be to identify an association between tgfβ1 genotype and disease. Although the experiments are more technically demanding, there have been a number of genetic studies that have attempted to determine whether polymorphisms in the tgfβ1 gene locus are associated with either myocardial infarction (a measure of plaque stability as well as plaque load) or angiographically defined luminal stenosis (a measure of plaque load). The first study, by Cambien et al40 in the ECTIM cohort, found an association between polymorphisms in the TGF-β1 signal peptide region of the gene and myocardial infarction but no association with angiographically defined arterial stenosis. A follow-up study confirmed the lack of association between several tgfβ1 polymorphisms and angiographically defined disease.41 However, a recent study in Japanese men42 found a larger association between tgfβ1 polymorphisms and myocardial infarction than that originally reported by Cambien. Taken together, these studies provide tentative support for the protective cytokine hypothesis in humans: polymorphisms associated with lower TGF-β1 secretion are over-represented among men with myocardial infarction in several populations.43 The lack of association with angiographically defined vessel stenosis41 may suggest that in humans, the role of TGF-β is more associated with the switch between stable matrix-rich lesions and unstable inflammatory lesions (the “second hit” of reduced TGF-β) than in determining the total atherosclerotic plaque load (the “first hit”).17,19,20

Unfortunately, a more clear-cut resolution of this question is unlikely to come from genetic studies of the tgfβ1 gene. Because TGF-β1 is involved in such a wide range of physiological and pathological functions, polymorphisms or mutations that caused significant changes to the temporal, spatial, or quantitative production of TGF-β would likely have effects that manifested themselves much earlier in life than atherosclerosis. Unlike in mice, in which definitive experiments were possible, it is likely that a fuller understanding of the role of TGF-β in human cardiovascular disease will have to come from the weight of evidence yielded by an array of experimental approaches.

**How Important Is TGF-β Signaling in Atherogenesis?**

Recent studies from a range of groups have confirmed that in rodents, suppression of TGF-β activity results in increased lipid lesion deposition during early lesion development and a switch from a stable, matrix-rich plaque phenotype to an unstable, proinflammatory plaque at risk for rupture.19-21 The available evidence suggest that similar pathways are likely to
A common consequence of a range of environmental and myocardial infarction. Effective preventative therapy will depend on targeting each of the pathways contributing to the risk for a given individual.

It is no longer sufficient merely to ask whether defects in particular pathways might contribute to the an increased risk for myocardial infarction. Instead, we need to ask how much of the variation in susceptibility to myocardial infarction across the human population can be attributed to each of these pathways. It is likely that for different individuals, different pathways may dominate (Figure 3). For some individuals, such as those with familial hypercholesterolemia caused by genetic defects in the LDL receptor protein and some individuals with less severe lipoprotein metabolism defects, the lipoprotein trafficking and metabolism defect may be the dominant cause of the increased risk of myocardial infarction by increasing the atherosclerotic burden. For others, increased susceptibility to intravascular coagulation and imbalance between thrombotic and fibrinolytic cascades will conclusively determine the relative importance of the LDL receptor protein and some individuals with less severe lipoprotein metabolism defects, the lipoprotein trafficking and metabolism defect may be the dominant cause of the increased risk of myocardial infarction by increasing the atherosclerotic burden. For others, increased susceptibility to intravascular coagulation and imbalance between thrombotic and fibrinolytic cascades.

It is no longer sufficient merely to ask whether defects in particular pathways might contribute to the an increased risk for myocardial infarction. Instead, we need to ask how much of the variation in susceptibility to myocardial infarction across the human population can be attributed to each of these pathways. It is likely that for different individuals, different pathways may dominate (Figure 3). For some individuals, such as those with familial hypercholesterolemia caused by genetic defects in the LDL receptor protein and some individuals with less severe lipoprotein metabolism defects, the lipoprotein trafficking and metabolism defect may be the dominant cause of the increased risk of myocardial infarction by increasing the atherosclerotic burden. For others, increased susceptibility to intravascular coagulation and imbalance between thrombotic and fibrinolytic cascades will conclusively determine the relative importance of the LDL receptor protein and some individuals with less severe lipoprotein metabolism defects, the lipoprotein trafficking and metabolism defect may be the dominant cause of the increased risk of myocardial infarction by increasing the atherosclerotic burden. For others, increased susceptibility to intravascular coagulation and imbalance between thrombotic and fibrinolytic cascades.

Acknowledgments
I am grateful to Profs Jim Metcalfe and Peter Weissberg (University of Cambridge, UK) for their support during the formulation of the protective cytokine hypothesis, and to Dr David Mosedale for helpful discussions over many years. D.J.G. is a British Heart Foundation Senior Research Fellow.

References

Figure 3. A 3-pathway model for atherogenesis. Epidemiology and reverse genetic approaches have unequivocally demonstrated that there are at least 3 major pathways (represented as colored circles) contributing to the risk of myocardial infarction. For some individuals, a major defect in a single pathway is dominantly responsible. For individual 1, a major defect in lipoprotein metabolism is the major contributor to their risk. For individual 2, a major defect in fibrinolysis might be responsible. We propose, however, that for most individuals with coronary artery disease (represented by individual 3), it is a combination of mild defects in all 3 pathways that results in high risk for myocardial infarction. Effective preventative therapy will depend on targeting each of the pathways contributing to the risk for a given individual.


Transforming Growth Factor β and Atherosclerosis: So Far, So Good for the Protective Cytokine Hypothesis
David J. Grainger

Arterioscler Thromb Vasc Biol. published online December 29, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2003/12/29/01.ATV.0000114567.76772.33.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/