**Introduction**

The earliest stage in the development of atherosclerosis in coronary arteries is believed to be altered function or injury of the endothelial cells lining the arterial lumen [1]. These changes cause local adhesion of various circulating cells and trigger the release of growth factors and chemokines, of which platelet-derived growth factor was a prototype [2]. In response to these factors, the contractile vascular smooth muscle cells (VSMCs) in the media of the artery migrate to the vessel surface and proliferate to form the early intimal lesion. In proliferating, the cells lose their major smooth-muscle-specific contractile proteins in a process of de-differentiation. It has been suggested, but not demonstrated, that only a minor sub-population of the VSMCs are capable of migration and proliferation, which might account for the monoclonal cell populations observed in most atherosclerotic plaques [3].

Abbreviations used: (h)VSMCs, (human) vascular smooth muscle cells; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; tPA, tissue plasminogen activator; TVD, triple vessel disease; NCA, normal coronary arteries.

The progression of the early lesion to an advanced, clinically significant plaque depends on a variety of genetic and lifestyle factors, reflected in the structural diversity of advanced plaques. However, infiltration of the lesion by circulating cells, particularly macrophages and lymphocytes, and the uptake of low-density lipoprotein (LDL) by macrophages to form foam cells, is a common feature of the advanced plaque. When the core of the plaque becomes necrotic, lipid is released from the foam cells to form lipid pools, with which calcium deposits are often associated. Rupture of the lesion results in thrombosis from the exposure of the highly thrombotic lipid core. Rupture frequently occurs at the shoulders of the plaque, at sites where high concentrations of macrophages and lymphocytes are found.

Blood risk factors associated with the development of advanced atherosclerosis have been identified by population studies. These risk factors include high concentrations of lipoprotein(a) [Lp(a)], which is elevated in about 20% of the population [4], and of LDL [5]. Lp(a) consists of an LDL particle with a molecule of the distinguishing protein, apolipoprotein(a) [apo(a)]. Recently it has also been found that a high concentration of plas-
minogen activator inhibitor-1 (PAI-1) in plasma is associated with increased risk of cardiovascular disease.

In addition to risk factors, population studies have also identified blood factors associated with protection against cardiovascular disease, including high-density lipoprotein [5]. Our own recent studies have led to a new working hypothesis for cardiovascular protection: that transforming growth factor-β (TGF-β) activity is a dominant inhibitor of atherosclerosis which must be suppressed before the various risk factors can drive the development of advanced disease [6-8]. This hypothesis was derived from three types of studies, which are outlined below.

**Effects of TGF-β and Lp(a) on the proliferation of human VSMCs in vitro**

TGF-β is synthesized by cells in a latent, propeptide form with no known biological activity and is activated proteolytically by plasmin to yield the active 25kDa homodimer [9]. Human VSMCs (hVSMCs) in culture secrete TGF-β in the latent form and also synthesize tissue plasminogen activator (tPA), mainly associated with the cell surface [10]. The tPA cleaves plasminogen present in the culture serum to give active plasmin, which in turn activates the latent TGF-β. The active TGF-β acts as an autocrine inhibitor of the proliferation of the cells. The mechanism of inhibition is unusual in that the cells continue to proliferate, but with a substantial extension of the G2 phase of the cell cycle [11]. This G2 extension is mediated by a delay in the transient cyclic AMP pulse which drives the entry of the cells into M phase. Lp(a) was found to relieve the inhibition of hVSMC proliferation by TGF-β by an indirect mechanism [6]. The relevant biochemical feature of Lp(a) is that apo(a) has an 80% sequence identity with the corresponding domains of plasminogen and consequently acts as a competitive inhibitor of tPA [12]. In culture, apo(a) or Lp(a) therefore block the activation of plasminogen to plasmin and hence the activation of latent TGF-β.

Based on this *in vitro* evidence, we proposed [6] that, since the proliferation of smooth muscle cells is a hallmark of atherosclerosis, Lp(a) might exert its atherogenic effect *in vivo* by inhibiting the activation of TGF-β and thus promote the proliferation of the VSMCs (Figure 1).

**TGF-β in the transgenic apo(a) mouse**

The transgenic apo(a) mouse provided an appropriate model in which to test *in vitro* the hypothesis linking apo(a) to inhibition of TGF-β activation and consequently to VSMC proliferation and lesion formation. The human gene for apo(a) is expressed in the apo(a) mouse under the control of the transferrin promoter to direct expression of the protein to the liver [13]. Since there is no murine homologue of the apo(a) gene, the apo(a) in the circulation of the mouse is entirely derived from the human transgene. The concentration of apo(a) in the circulation (typically ~6 mg/dl) is similar to the concentration in the normal human population. However, the human apo(a) in the mouse does not associate with murine LDL to form Lp(a), presumably because the appropriate cystine bond with the apoB 100 protein component of murine LDL cannot be formed [13]. The circulating apo(a) therefore accumulates without LDL in the walls of the coronary arteries and other blood vessels. Although apo(a) can be detected throughout the vessel wall, there are also focal points of very high concentrations of apo(a). When fed on a lipid-rich diet, the apo(a) mice develop lipid accumulations in the vessel wall at sites of high apo(a) accumulation, which result in coronary artery stenoses [13].

Techniques were developed to test each of the steps in the pathway linking apo(a) to inhibition of TGF-β activation (Figure 1) in aortic sections of apo(a) mice and their normal littermates not carry-
ing the apo(a) gene [7]. Plasmin was assayed by using fluorescein-labelled α,-antiplasmin, a highly specific inhibitor which binds covalently to plasmin but does not interact covalently with plasminogen or other proteins in aortic sections. To assay for plasminogen the same sections labelled with α,-antiplasmin were treated with excess tPA to convert the plasminogen to plasmin, and then relabelled with α,-antiplasmin labelled with rhodamine. Quantitative immunofluorescence showed that approx. 6% of the plasminogen present in sections from the normal litters was in the active plasmin form, whereas only 2% was active in apo(a) mouse sections. These data are therefore consistent with apo(a) causing inhibition of plasminogen activation [7].

The proportions of active and latent TGF-β in the aortic sections were also assayed by quantitative immunofluorescence techniques. The amount of active TGF-β was assayed using a truncated form of the type II TGF-β receptor labelled with fluorescein, which binds active but not latent forms of TGF-β. Combined active and latent TGF-β were assayed with an antibody having equal affinity for the active and latent forms. In the normal littermate mice, the TGF-β was predominantly in the active form (~90%) whereas in the apo(a) mouse aortic sections only ~40% of the TGF-β was in the active form. Thus the presence of apo(a) in the vessel wall is correlated with inhibited activation of TGF-β [7].

Another significant observation was that the activation of TGF-β was most severely inhibited at sites of high focal concentration of apo(a) in the vessel wall. At the same sites there was also evidence of strong activation of the VSMCs, marked by the expression of osteopontin, a protein associated with calcification in plaques [14,15]. This activation process occurred in apo(a) mice but not in their normal littermates, irrespective of whether the mice were on a lipid-rich or normal diet. Activation of the VSMCs is therefore a response to the presence of apo(a) in the vessel wall, rather than to the accumulation of lipid [7].

Taken together, these data provided powerful support for the hypothesis that in the vessel wall apo(a) inhibits the activation of plasminogen, and that the consequent inhibition of TGF-β activation results in local activation of the VSMCs.

**TGF-β and advanced atherosclerosis**

The agents used to assay TGF-β in the vessel wall can also be used as capture agents for ELISAs to measure the concentrations of active and latent TGF-β in serum or plasma. We found that the proportion of TGF-β in the active form was reduced by about 70% in the sera of apo(a) mice compared with their normal litters. These data suggested that analysis of TGF-β in human sera would be technically feasible.

TGF-β concentrations were compared in the group of patients with at least 50% stenoses of all three coronary arteries (triple vessel disease; TVD group) and a group of patients shown by angiography to have normal coronary arteries (NCA group) [8]. It was found that for both groups of patients the range of combined active plus latent TGF-β concentrations was wide, varying from less than 1 ng/ml to over 100 ng/ml. However, a subgroup of patients in the TVD group (11/31) had no detectable TGF-β by the ELISA (<1 ng/ml), whereas all (30/30) of the NCA group had at least 2 ng/ml TGF-β. When active TGF-β was assayed, there was a striking difference between the two groups. All of the NCA group had at least 2 ng/ml active TGF-β, whereas only two of the TVD group had detectable amounts of TGF-β (1 ng/ml) and the remainder (29/31) had no detectable active TGF-β (<1 ng/ml). There was therefore at least a 5-fold difference in the mean concentration of active TGF-β between the two groups, providing a much clearer distinction than for other risk factors [8].

Multiple regression analysis of the data revealed that the clearest independent correlations were obtained between the proportion of TGF-β in the active form and the concentrations of Lp(a) or PAI-1. Since PAI-1 and Lp(a) act as independent inhibitors of tPA in vitro, PAI-1 can readily be accommodated in the model linking the activation of TGF-β to atherosclerosis (Figure 1). Two other interesting correlations were observed: women were substantially more effective at activating the TGF-β in their serum than men, and patients in the NCA group taking aspirin had higher levels of TGF-β (active or latent) than patients not taking aspirin. None of the other medications taken had a significant effect on TGF-β. We therefore examined the effect of aspirin on TGF-β synthesis by VSMCs and found that it stimulated TGF-β production by at least 10-fold, with an ED₅₀ of ~3 μM. No other drugs being taken by the patient groups affected the production of TGF-β by VSMCs in vitro.

**Significance of the TGF-β correlation with atherosclerosis**

The key question raised by the clear differences in active TGF-β between the TVD and NCA groups is whether a very low active TGF-β concentration is merely a correlate of the disease, for example a...
response to a high plaque load, or whether it reflects a causative relationship between TGF-β and the generation of the disease, as we have hypothesized [6–8]. The diagnostic and prognostic value of the correlation is therefore the focus of current work. We have found that in nominally healthy groups who donated blood samples, the same correlations between TGF-β activation, Lp(a) and PAI-1 were obtained as for the patient groups, strongly indicating that the same factors determine activation of TGF-β in the population at large as in the patient groups [8]. We have also found that the proportion of men in apparently healthy donor groups with no detectable serum TGF-β (<1 ng/ml) is substantially higher than the corresponding proportion of women [8]. A comparative study of donors from Toulouse and Belfast also suggests that the concentration of active TGF-β in serum reflects the difference in incidence of coronary heart disease in the two populations (D. J. Grainger, J. C. Metcalfe and Norman R. Williams, unpublished work). Large-scale prospective studies of the predictive value of TGF-β measurements are therefore planned.

Another approach to evaluating the role of TGF-β in atherogenesis is to determine whether interventions which increase the concentration of active TGF-β in the vessel wall affect lesion development. In the apo(a) mouse this can be achieved either by the production of double transgenics [e.g. by crossing apo(a) mice with TGF-β overexpressers] or by gene therapy approaches using viral constructs to deliver TGF-β genes. For human studies, the VSMC culture system can be used to screen commonly used drugs for their ability to stimulate TGF-β production. Of the drugs found to have this effect, the anti-oestrogen, tamoxifen, is the most potent [16]. Tamoxifen is currently being tested in the apo(a) mouse model to determine if it will prevent lesion development. Small-scale studies to determine whether tamoxifen causes elevation of TGF-β in the serum of patient groups have also been initiated. There is evidence from a trial of tamoxifen as an adjuvant therapy for breast cancer that it has a substantial cardioprotective effect against myocardial infarct [17], but the mechanism of this effect has not been established. However, it is clear that the hypothesis linking the loss of active TGF-β to the development of atherosclerosis is accessible to intervention studies both in animal models of the disease and in clinical studies.

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