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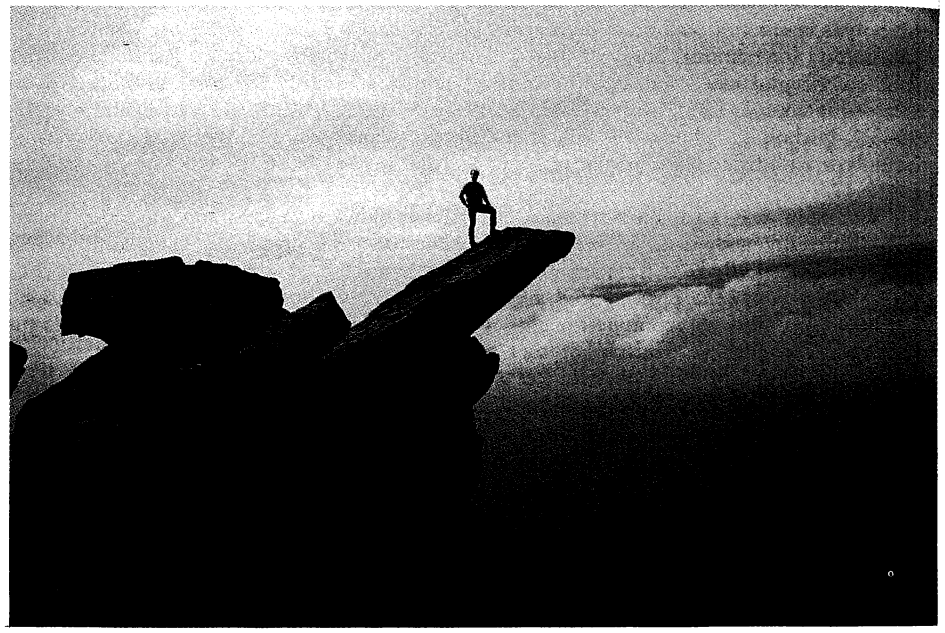

The Lover's Stone, St Kilda. Tradition has it that suitors on the island had to prove their manhood by balancing precariously on one foot on the edge of this 300 foot sheer drop to the sea. The resident population of the island was evacuated in 1930 and it is now owned by the National Trust for Scotland. (Photograph by David H. A. Boyd).

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MOLECULAR APPROACHES TO CARDIOVASCULAR DISEASE: A GLIMPSE OF THE FUTURE*

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The impact of molecular biology on clinical cardiological practice has so far been modest. However, research in laboratories throughout the world is focused on identifying genes responsible for the development or progression of cardiovascular disease with the realistic expectation that their findings will be translated into novel therapeutic strategies in the not too distant future. As in all other branches of medicine, it is the relatively rare single gene defects which have proved most amenable to molecular study, hence the genetic mutations responsible for diseases such as Marfan's syndrome, familial hypercholesterolaemia, hereditary haemorrhagic telangiectasia and hypertrophic cardiomyopathy have been identified. However, whilst knowledge of the genetic defects which result in these rare conditions has provided important insights into the pathogenesis of these and commoner related diseases and has significantly improved diagnostic accuracy, it has not yet led to effective therapeutic advances. Ultimately, cure of these conditions requires replacement of the defective gene by some form of gene therapy, an area of clinical science only in its infancy and likely to be confined for the forseeable future to a few specialist centres.

Of more interest to most practising cardiologists are the genes which cause common cardiovascular disorders such as coronary artery disease, which almost certainly results from the interaction between a number of 'normal' gene products and environmental factors such as diet and smoking. The future management of coronary disease therefore lies in the identification of genes whose products contribute to the progression or stabilisation of atherosclerosis with the aim of manipulating their expression to modify disease progression. Ultimately, this is likely to require the use of novel therapeutic agents which target enhancer or suppresser sequences in the non-coding region of candidate genes; an approach which could be termed 'pharmacological gene therapy'. Although this may seem somewhat far fetched, it is important to recognise that many drugs in current use achieve some of their therapeutic effect indirectly through this mechanism. For example, in addition to reducing cholesterol synthesis, HMG CoA reductase inhibitors also up-regulate expression of LDL receptors, thereby targeting the fundamental genetic defect in some forms of hypercholesterolaemia. Elucidation of the mechanism by which this is achieved may lead to drugs which directly modify expression of the LDL receptor.

It is likely that over the next decade new therapeutic approaches to the management of coronary heart disease will evolve from today's laboratory research. The aim of this article is: firstly, to outline some of the molecular approaches being used to identify potential candidate genes and to determine their contribution to vascular disease; secondly, to introduce molecular strategies

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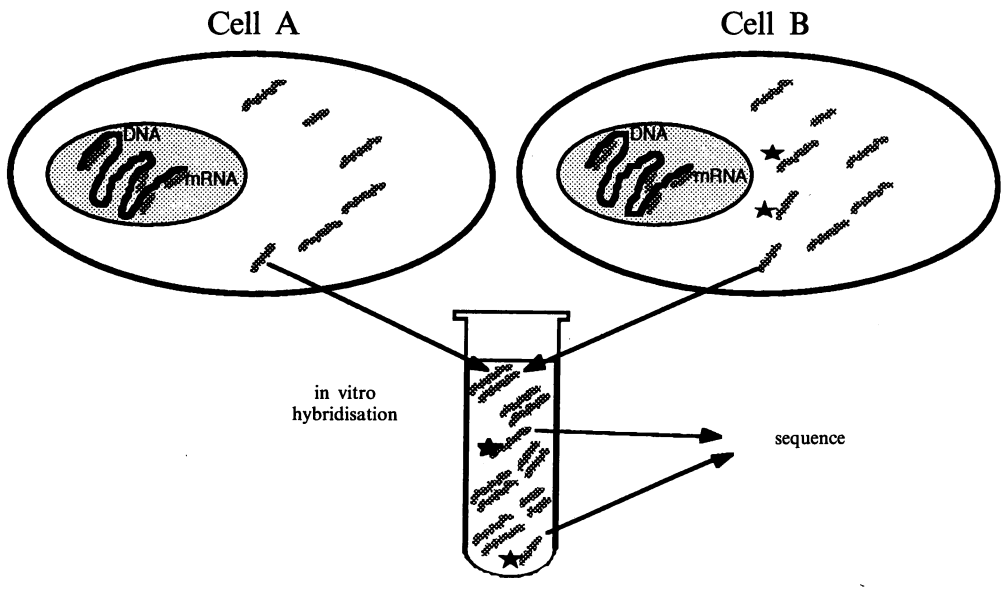

FIGURE 1
Identification of differentially expressed genes. mRNA is isolated from each cell type and processed for *in vitro* hybridisation to bind out identical species. mRNA sequences which do not have a 'partner' can be isolated and sequenced.

aimed at repairing the damaged myocardium following infarction; and finally to touch on the potential impact of molecular biology on organ transplantation. These concepts have been chosen for discussion because they are either close to, or already undergoing, clinical evaluation and may therefore herald imminent changes in clinical practice.

IDENTIFICATION OF DISEASE-ASSOCIATED GENES IN VASCULAR DISEASE

The 'response to injury' hypothesis of atherogenesis of Ross and colleagues in the 1970s proposed that inappropriate proliferation of vascular smooth muscle cells was a key event in the development of an atherosclerotic plaque.[1] This hypothesis was supported by the proposal that re-stenosis following angioplasty was caused by over-exuberant proliferation of intimal vascular smooth muscle cells following balloon injury. Numerous laboratories around the world have therefore used proliferating vascular smooth muscle cells in cell culture to model the atherosclerotic process. Although most cells in the body contain a full complement of an individual's DNA, it is the subpopulation of genes which are transcribed to messenger RNA (mRNA), and then translated into proteins, which determine the characteristics, or phenotype, of a particular cell. Therefore, by isolating the mRNA from one cell type and comparing it with mRNA from another, molecular biologists can identify genes which dictate the phenotype of different cells (Fig 1).

Using a variety of molecular biological techniques, a number of genes have been identified which are differentially expressed in proliferating vascular smooth muscle cells and which may, therefore, be involved in the development of atheroma. For example, in our own laboratory we have identified two such genes

as coding for osteopontin and matrix Gla protein,[2] both bone-associated genes which had not previously been known to be expressed by vascular cells. Using the technique *in situ* hybridization we were able to confirm that both genes were expressed by cells within atherosclerotic plaques but not by normal smooth muscle cells in healthy arteries.[3] Furthermore, the genes were most highly expressed at sites of vascular calcification. We and others have since shown that, under certain circumstances, vascular cells can express a number of other genes more commonly expressed by bone cells. These results imply that the process of vascular calcification, which has traditionally been thought of as a passive terminal event in necrotic tissue, occurs by a regulated process similar to that occurring in developing bone and which may therefore be amenable to intervention to prevent its progression. Progress in this novel area of research may have important implications, not only for the treatment of vascular disease, but also possibly for the future medical management of degenerative calcific valve disease where similar genes are expressed.[4]

DETERMINING THE ROLE OF CANDIDATE GENES

Having identified genes whose products might play a role in vascular disease, it is important to determine their role in vascular pathology. This requires the ability to both over-express and to inhibit expression of the gene of interest in models of vascular disease and ultimately in man.

Enhancing gene expression

Some of the best examples of over-expression studies in vascular pathology come from the laboratory of Elizabeth and Gary Nabel. In a series of elegant experiments they have delivered genes for different vascular smooth muscle growth factors into the femoral artery of a pig, using a double balloon catheter. By this approach they have shown that platelet-derived growth factor causes accumulation of vascular smooth muscle cells on the intimal aspect of the internal elastic lamina to create a 'neointima'.[5] Fibroblast growth factor-1 causes intimal smooth muscle cell accumulation and also stimulates development of new blood vessels within the neointima.[6] Transforming growth factor β causes the accumulation of fewer smooth muscle cells with the elaboration of large quantities of extracellular matrix.[7]

Studies of this nature serve two useful purposes. Firstly, they provide important information on the probable role of individual growth factors which are often co-expressed at the sites of vascular injury, and secondly, they demonstrate the feasibility of potentially treating vascular lesions by local gene therapy.

Over recent years, numerous laboratories have been trying to introduce new genes into the vascular wall of laboratory animals as a prelude to human studies with only limited success. The main obstacles to successful gene transfer are delivery of the gene of interest and regulation of its expression in the target cell. For successful cell transduction, foreign DNA must be delivered across the cell membrane, translocated to the nucleus and transcribed into mRNA. Genes delivered as naked (plasmid) DNA can successfully transduce cells, however this is an extremely inefficient method of gene transfer since the DNA is poorly taken up and readily destroyed. More efficient gene delivery is achieved by complexing the DNA with liposomes which fuse with the cell membrane and deliver the DNA to the cytoplasm, but the lipids used can be toxic and efficiency *in vivo*

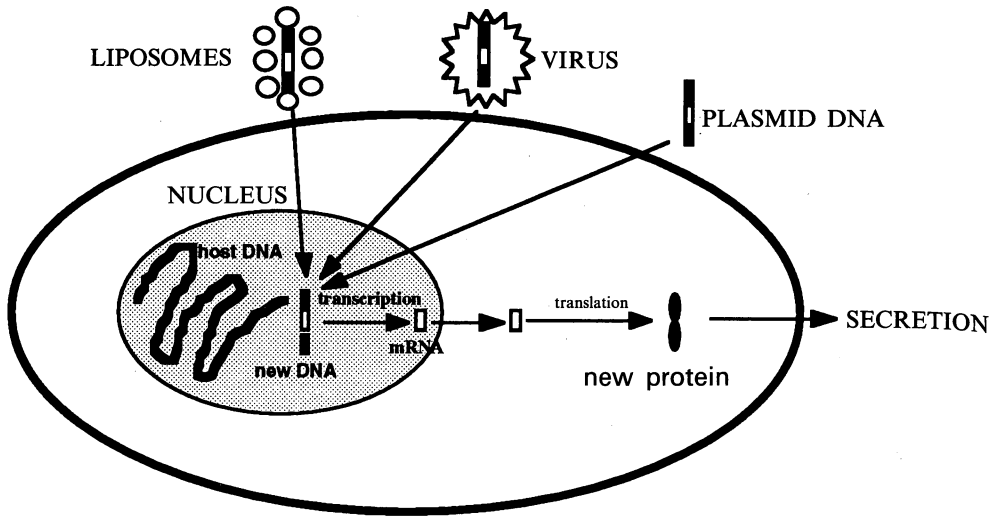

FIGURE 2
Methods for introducing new genes into cardiovascular cells.

remains disappointingly low. The most efficient vectors for delivering foreign DNA to the nucleus are replication-deficient viruses which have been genetically modified so that they carry the new DNA in place of the DNA coding for the genes required for their own replication. As such, they are simply DNA delivery vectors. Retroviruses efficiently deliver new genes to the nucleus, but only in proliferating cells, whilst non-proliferating cells are best transduced by replication-deficient adenoviruses (Fig 2). However, so far, successful vascular cell transduction *in vivo* has only been achieved by using very high concentrations of virus which cause local immunological reactions which may have direct effects on vascular pathology.

Even if successfully delivered to the nucleus, a foreign gene will not be transcribed into mRNA if the new DNA does not incorporate an appropriate promoter sequence. Ideally, this should be a sequence known to be uniquely activated in the target cell, but so far few cell-specific promoter sequences have been identified. Therefore, gene transfer constructs in present use incorporate a viral promoter (cytomegalovirus immediate early promoter or the Rouse sarcoma virus long terminal repeat) which is thought to be active in most cells. However, recent evidence from our own laboratory indicates that these promoters are not optimally active in human vascular cells.[8] Nevertheless, continued research will inevitably identify cell-specific promoters, and newer viral vectors are being developed which carry larger pieces of 'new' DNA and fewer genes coding for antigenic viral proteins. Therefore, despite the present obstacles, it is likely that gene therapy to modify vascular disease will become a reality in the not too distant future.

Inhibition of gene expression

The contribution of a particular gene product to the pathogenesis of a disease is best evaluated by neutralising its effects. Where the product is a receptor ligand, this is achieved by designing a pharmacological receptor antagonist. However, if

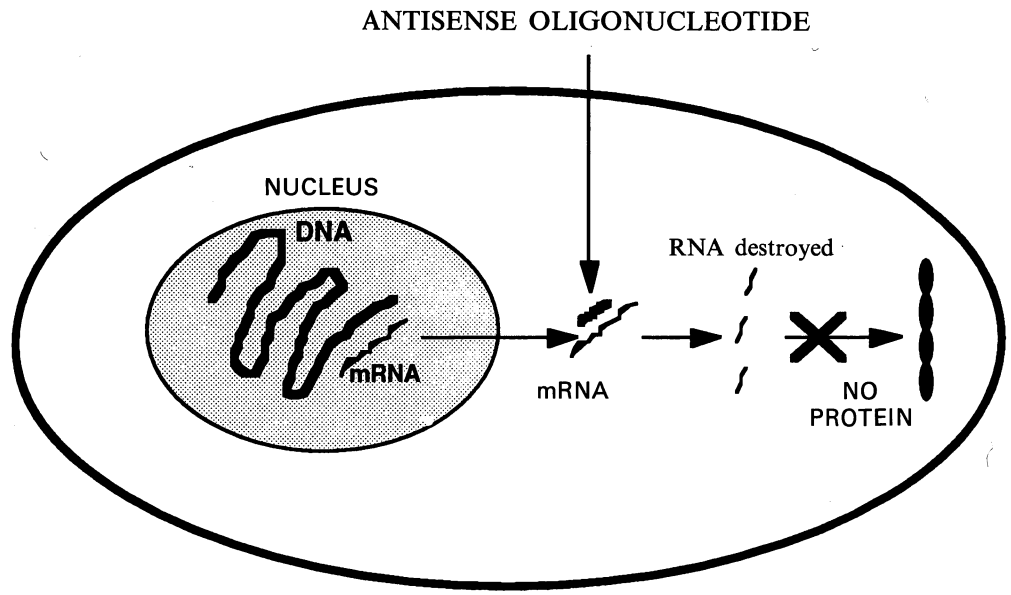

FIGURE 3
Use of antisense oligodeoxynucleotides to inhibit gene expression.

the gene product is an intracellular signalling or structural protein, this approach may not be appropriate. Therefore, attention has turned towards developing strategies to inhibit transcription or translation of the candidate gene.

Attempts to inhibit gene expression in vascular disease so far have most commonly relied on the use of antisense oligodeoxynucleotides (ODNs) (Fig 3). Antisense ODNs are short sequences of DNA which are synthesised to be complementary to a segment of mRNA transcribed from the target gene. In theory, the antisense ODN hybridises uniquely with the target mRNA in the cytoplasm leading to its rapid destruction by RNA degrading enzymes. The mRNA therefore fails to be translated into protein. Because of the inherent instability of antisense ODNs, they are usually chemically modified to make them resistant to extracellular and intracellular destruction. Several groups have now reported successful inhibition of neointima formation following balloon-induced injury in a variety of laboratory animal models using antisense ODNs to cell cycle genes known to be essential for successful passage of vascular smooth muscle cells through the cell cycle.[9,10] However, a number of concerns have been raised about the validity of this approach to suppress gene expression.[11] For example, many investigators have found that sense ODNs, which should have no effect on the target mRNA, also have a significant biological effect, implying an, as yet poorly understood, non-specific effect of ODNs on cellular processes. Despite these reservations, clinical trials of locally delivered antisense ODNs to inhibit re-stenosis following angioplasty are either in progress or soon to be launched both in the UK and the USA.

MYOCARDIAL REPAIR

Although new therapeutic strategies aimed at inhibiting progression or even causing regression of atherosclerosis will emerge over the next decade, they are

unlikely to have a significant impact on the incidence of myocardial infarction for a considerable time. The main consequence of myocardial infarction is myocyte death with subsequent pump failure, and it is well established that mortality following infarction is directly related to the degree of myocardial damage. At present there is no satisfactory means of restoring myocardial function following infarction short of transplantation. Myocardial repair would require renewed perfusion of infarcted myocardium and the restoration of contractile muscle cells, neither of which is achievable at present. However, each of these goals is theoretically achievable and already significant progress has been made along the road to myocardial repair in a number of molecular biology laboratories.

Restoring perfusion

Over the past twenty years a large number of growth factors have been identifed which are mitogenic for vascular cells. One of these, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, binds uniquely to vascular endothelial cells inducing their proliferation. Studies by Isner and colleagues in Boston showed that infusion of recombinant VEGF protein or plasmid DNA encoding VEGF into the ischaemic hind limb of a rabbit accelerated collateral vessel formation.[12] They also showed that VEGF accelerated endothelial repair, with reduced neointima formation, following ballon-induced injury to the rat carotid artery.[13] The promising results of these experiments have led to approval of the first gene therapy protocols, using the gene encoding VEGF, to treat severe peripheral vascular disease and to inhibit re-stenosis following femoral angioplasty in man.[14]

Regardless of the outcome of these pioneering studies, it is likely that knowledge of the genes responsible for neovascularisation of ischaemic tissue will ultimately lead to therapeutic programmes aimed at revascularising ischaemic myocardium. As uncontrolled angiogenesis in healthy tissues with its attendant risks of haemorrhage and thrombosis would be undesirable, an element of selectivity for ischaemic tissue will be required. In theory this could be achieved by using an ischaemia or hypoxia-responsive promoter to drive expression of the angiogenic gene(s). Erythropoeitin is a protein whose production is known to be exquisitely sensitive to ambient oxygen tension. A sequence has already been identified in the promoter for the erythropoeitin gene which is bound by specific hypoxia-responsive transcription factors whose transcription is finely regulated by oxygen pressure.[15] It is feasible therefore that constructs could be engineered which would permit transcription of angiogenic genes only in ischaemic tissues, potentially leading to improved perfusion of infarcted or ischaemic myocardium.

Restoring muscle

Unlike skeletal and smooth muscle, adult cardiac myocytes lack the ability to replicate, thus myocardial infarction causes permanent muscle loss. At present, cardiac myoplasty is the only therapeutic surgical option for improving myocardial function. This involves wrapping a sheet of skeletal muscle from the chest wall around the damaged heart. However, because skeletal muscle fatigues rapidly when stimulated to contract repetitively, it is also necessary to induce in it a change in the ratio of fast to slow muscle fibres by a programme of repetitive electrical stimulation before surgery—an example of a therapeutically-induced change in gene expression. Despite these efforts, surgical myoplasty has not established itself as a major therapeutic advance.

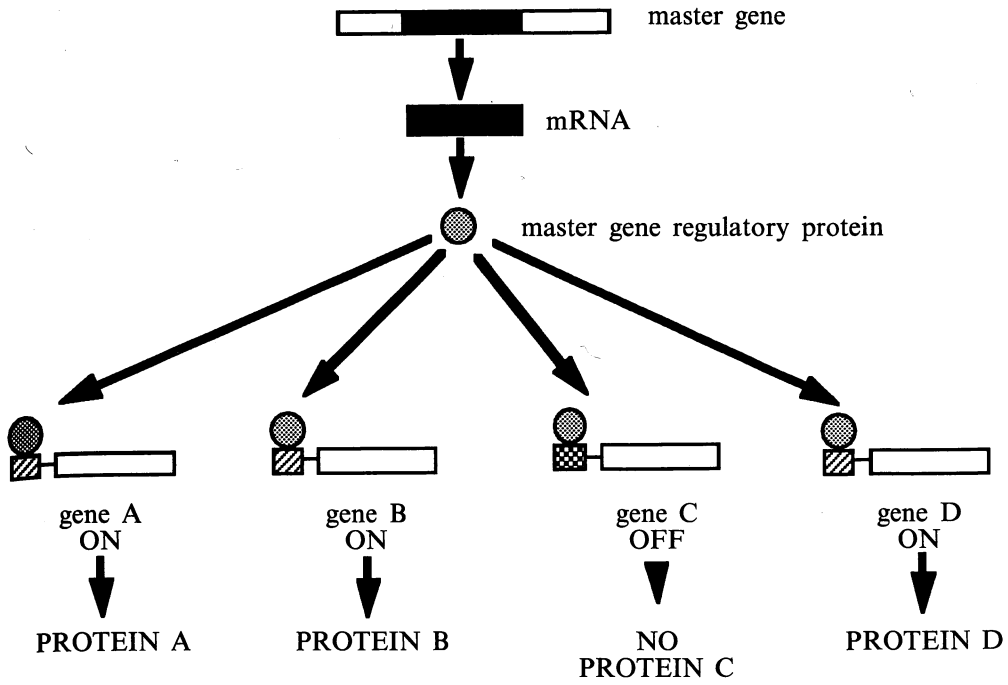

FIGURE 4

The concept of the master gene regulator. The master gene product binds to enhancer sequences in genes for proteins A, B and C and to a repressor sequence in the gene for protein C, thereby dictating the overall pattern of gene expression and therefore cell phenotype.

Studies of the molecular events leading to the differentiation of skeletal myoblasts have identified a class of transcription factors called 'master gene regulators'. These transcription factors bind to the upstream sequences of several genes causing inhibition of expression in some and enhanced expression in others (Fig 4). The net effect is the expression of a repertoire of genes which dictates a particular phenotype. In skeletal muscle such a gene is known as myo-D.

When myo-D is expressed in immature myoblasts the cells differentiate into mature skeletal muscle cells and express all the genes required for myotube formation and contraction.[16] Importantly, if myo-D is transfected into non-muscle cells such as fibroblasts, they also express skeletal muscle proteins and form myotubes. Thus by molecular manipulation a fibroblast can be turned into a muscle cell. As yet, the myocardial equivalent to myo-D remains to be discovered. However, once cardiac specific transcription factors have been identified, it would be feasible to transfect cardiac fibroblasts within infarcted tissue with the appropriate myogeneic genes. Even now, transfection of cardiac fibroblasts with myo-D induce skeletal myoblast formation within infarcted myocardium, bringing the prospect of a 'cellular myoplasty' or myocardial repair closer to reality.

XENOTRANSPLANTATION

For the immediate future there is no satisfactory remedy for the badly damaged myocardium, regardless of the aetiology, and the only realistic therapeutic prospect for many patients is organ transplantation. Orthotopic cardiac transplan-

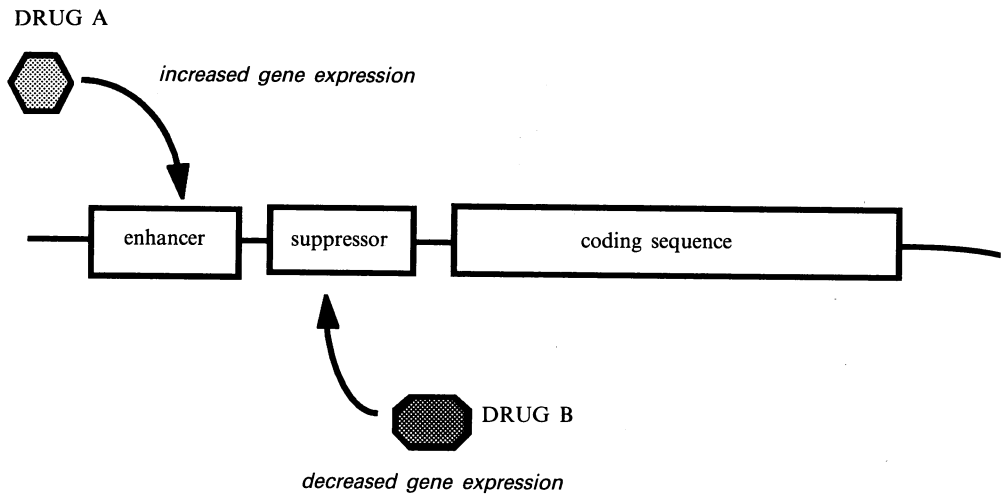

FIGURE 5
The concept of pharmacological gene therapy to enhance or suppress expression of a disease-associated gene.

tation has become a routine procedure with a steadily improving outcome. However, the main obstacle to the widespread use of this treatment is the limited availability of suitable donor organs. So far, mechanical devices have proved cumbersome and unacceptably thrombogenic. Hence transplant scientists have turned to other species as a potential source of organs.

Early experiments disappointingly showed that organs transplanted across species were subject to the phenomenon of hyperacute rejection in which intravascular thrombosis lead to infarction within minutes of transplantation. This was due, not to antibodies, but to complement-mediated vascular endothelial cell destruction,[17] a process which is prevented in homografts by the presence of a surface glycophospholipid called 'decay accelerating factor' (DAF) which prevents the development of complement attack complexes in a species-specific manner. These findings suggested that if animal organs could be induced to express human DAF (hDAF) on their endothelial cells, they might be protected from hyperacute rejection when exposed to human blood. Subsequent studies proved this to be so[18] and paved the way for the development of a colony of transgenic pigs expressing hDAF. Recent experiments in which hearts from these pigs were transplanted into immunosuppressed Cynomolgus monkeys shows that the hearts do not undergo hyperacute rejection and can function for several weeks without evidence of rejection (D. White, personal communication). These results have been sufficiently encouraging for plans to be made for the first human transgenic xenotransplant to take place in the near future.

CONCLUSIONS

The full impact of the molecular biological revolution has yet to be felt in cardiovascular medicine. Nevertheless, the relentless search for disease-associated protective genes and the continual refinement of techniques for controlling their expression *in vivo* will undoubtedly lead to novel approaches to the management of rare and common cardiovascular diseases in the not too distant future. Protocols for direct gene transfer and antisense inhibition of gene expression are already undergoing clinical evaluation, and the use of genetically engineered organs for transplantation will soon move from the realms of science fiction to science fact. As with all new technologies, initial attempts at therapy are necessarily cumbersome and unrefined. However, as more is learned about the intracellular signals which orchestrate specific gene expression, new pharmacological approaches aimed at manipulating target genes are likely to evolve, bringing 'pharmacological gene therapy' within the realms of the practising cardiologist (Fig 5). Inevitably, cardiologists of the future will need to understand the mechanisms which govern the regulation of gene expression in much the same way as today's cardiologists understand the actions of receptor blocking drugs.

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