

# What are the prospects for gene therapy in atherosclerosis?

**E. Arbustini, MD**

*Istituto di Anatomia Patologica - Laboratorio di Patologia Cellulare - IRCCS Policlinico San Matteo - Pavia - ITALY*

*Although gene therapy strategies are applicable to genetically determined, preferably monogenic disorders, recent advances regarding multifactorial disorders, such as atherosclerosis, are promising. Gene therapy strategies have been developed against genetically determined risk factors, such as familial hypercholesterolemia and familial dysbetalipoproteinemia, as well as to stimulate factors that protect against, or reverse, the disease through over-expression of protective lipoproteins, such as high-density lipoproteins (HDL). For gene polymorphisms associated with increased risk of atherosclerosis, genetically guided drug therapy (eg, angiotensin-converting enzyme (ACE) inhibitors in patients with DD ACE genotype) is a distinct possibility. Other genetic determinants need to be addressed, eg, hyperhomocysteinemia. Future gene therapy strategies for atherosclerosis will likely evolve in tandem with the emergence and elucidation of individual determinants.*

Conventional prerequisites for gene therapy are that the given disorder be genetically determined, preferably monogenic, and possibly transmitted as an autosomic recessive trait. If these prerequisites were valid, atherosclerosis would appear to be inadmissible as a suitable condition for gene therapy: it is a multifactorial, multidistrictual, and multifocal disease.<sup>1</sup> Moreover, it is accompanied by such a variety of morphological-structural characteristics that it suggests a "spectrum" of disorders rather than a single disease. Despite these unpromising premises, recent research has focused precisely on atherosclerosis as a candidate for gene therapy. This focus must, however, be set within a realistic and well-proportioned context.

Gene therapy has attempted to address those illnesses that determine an increase in the risk (metabolic, for example) of atherosclerosis. In particular, attention in the past 20 years has prioritized the risk factors for atherosclerosis, prominent among which is dislipidemia, and primary prevention strategies have produced encouraging results; the Scandinavian Simvastatin Survival Study (4S) study is but one example.<sup>2</sup> It is obvious that patients submitted to a variety of therapies (diet, lifestyle, drugs, gene therapy) have necessarily varying profiles, and that while in theory drug and lifestyle therapies are applicable to all dyslipidemic

patients, the same is not true for gene therapy; the latter requires both that the genetic substrates of the illness itself be known, and that other forms of therapy prove ineffective. Accordingly, only a very small proportion of dyslipidemic patients may be submitted to gene therapy, and the same holds for hypertension and diabetes patients:

*future strategies will rely on gene therapy to the extent that research identifies the gene that is responsible for the "risk factors," so that only a minority of patients will likely be suited to such therapy.*

However, these minorities are becoming increasingly numerous, and at the same time our understanding is progressing. Moreover, molecular screening techniques for genetically determined risk factors are now easily transferable to large populations. Taken together, these various developments open up interesting perspectives, not least in the case of multifactorial diseases such as atherosclerosis.

## **GENE THERAPY AND ATHEROSCLEROSIS**

Gene therapy can be defined as the replacement of a deficient gene product, or as the correction of an abnormal gene.<sup>3</sup> All current programs focus on somatic cell gene therapy; there is universal agreement that germ line gene



therapy is ethically unacceptable. The technical, therapeutic, ethical, and safety aspects of gene therapy programs are overseen by regulatory bodies.<sup>4</sup> The prerequisites for gene therapy strategies are that the gene involved should have been cloned, that target cells (ie, those which are to be treated) are identifiable and have a reasonable life span, and that a proper vector (viral or physical) for the therapeutic gene is available. *Viral* vectors include: retroviruses in packaging cell lines; stable adenoviruses (or adeno-associated viruses), which suit treatment that targets specific tissues, which also infect nondividing cells, and which carry large DNA segments; herpesviruses, which can target gene therapy, for example to nervous cells.

*Physical* vectors include liposome-mediated DNA transfer (possible future microchromosomes), receptor-mediated endocytosis, and antisense oligonucleotides, which bind to specific mRNAs. The therapeutic gene can be transferred with "in vivo" or "ex vivo" procedures, and the target organs/tissues can be reached with systemic injection or local delivery systems. Originally, monogenic disorders inherited as a recessive trait (eg, cystic fibrosis) were the ideal candidates for gene therapy.<sup>5</sup> Recently, atherosclerosis has been included in the list of treatable diseases: specifically, therapeutic choices have widened because it is now possible to target strategy to genetically determined risk factors, such as familial hypercholesterolemia (FH),<sup>6,7</sup> rather than to the fully established disease itself.

### **RISK FACTORS FOR ATHEROSCLEROSIS**

A series of so-called "risk factors" are known to be associated with an increased risk of developing

atherosclerosis; these include dyslipidemia, diabetes, hypertension, and cigarette smoking; a family history of ischemic heart disease (in relatives <55 years) is a risk factor for acute coronary events (which affect a minority of subjects suffering from atherosclerosis), rather than for the atherosclerosis itself. Minor factors include lifestyle-related factors, such as lack of physical activity, stress, and so on. Other conditions, most of them genetically determined, have been suggested to be associated with an increased risk of developing atherosclerosis (angiotensin-converting enzyme insertion/deletion polymorphism, angiotensin gene polymorphism, hyperhomocystinemia), but need further confirmation before entering the list of proven risk factors for atherosclerosis. Among the "so-called" risk factors, some (hypercholesterolemia, hypertension, diabetes) may be genetically determined<sup>8</sup>; current gene therapy strategies are mostly addressed to such factors (for example FH).<sup>7,9</sup> However, novel developing fields of investigation explore the possibility of providing protection against the disease by increasing the levels, for example, of circulating "benign" lipoproteins (high-density lipoproteins).

Finally, despite the numerous recent advances in the risk factor field, it is likely that the list of such factors is still incomplete: there are subjects whose profiles lack not only the so-called "risk factors," but also all evidence of any known condition predisposing to the disease. It follows that there must be "risk factors," or promoting or favoring conditions, that have yet to be identified. Our knowledge of the genetic and molecular mechanisms, and of the molecular basis of atherosclerosis, is still far from complete; accordingly,

gene therapy strategies will need to evolve in tandem with the progressive emergence and elucidation of each single determinant.

Other strategies should ideally aim at preventing the development of the disease, making arterial wall cells (eg, endothelial cells) resistant to the combined effect of multiple risk factor-related damage.

This approach is motivated by the fact that the early key pathogenetic event of atherosclerosis is endothelial cell damage, which is accompanied by loss of relaxing properties, increased permeability to circulating molecules, increased adhesiveness to circulating cells, and loss of antithrombotic properties.<sup>1</sup> Once endothelial cell functional and anatomical integrity is lost, circulating molecules and cells have access to the vessel wall, internal elastic lamina disruption opens the way for smooth muscle cell migration from the tunica media to the subendothelial spaces, and the early nucleus of the lesion takes form and progresses with differing and unpredictable rates at multiple vessel sites. However, although genetically modified endothelial cells can be introduced into vascular segments via localized intravascular delivery devices (balloons, stents), current gene transfer into arterial wall cells applies almost exclusively to ideal monofocal lesions in which a unique cell type (smooth muscle cells) is the major determinant of the given lesion, such as restenosis.

### **GENETICALLY DETERMINED "RISK FACTORS" FOR ATHEROSCLEROSIS POTENTIALLY SUITED TO GENE THERAPY STRATEGIES**

The following deals with current knowledge on the genetic basis of some risk factors that are already

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recognized as suited to gene therapy strategies, and of other genetically determined/influenced risk factors that may be suitable for future gene therapy research.

• **Familial hypercholesterolemia (FH)**

is the commonest single gene disorder in Western society. It is inherited as an autosomal dominant trait, with heterozygotes numbering about 1 in 500 and homozygotes about 1 in a million. It is estimated that 1 in 20 people with early coronary artery disease are heterozygotes. The disorder is phenotypically characterized by elevated cholesterol levels (with a high risk of early coronary artery disease development) and xanthomata (subcutaneous deposition of lipids). The high cholesterol levels are due to high levels of low-density lipoproteins (LDL), whose increase is a consequence of “deficient or defective” function of the LDL receptor. Homozygotes have: (i) little or no receptor activity; (ii) increased levels of LDL (4 times the normal); (iii) premature coronary artery disease; and (iv) a high risk of early death related to ischemic heart disease. Receptor-negative mutations completely eliminate the receptor function, and are associated with a devastating course of coronary artery disease, while receptor-defective mutations partially inactivate receptor activity, and are associated with less severe hypercholesterolemia and with later-onset coronary artery disease.<sup>8</sup> FH was identified as an ideal disorder for gene therapy because: (i) the genetic defects (mutations in the LDL receptor gene) were known; (ii) the defect had a severe phenotype, especially in homozygotes, which are refractory to traditional forms of therapy; (iii) it is possible to follow the outcome of gene therapy through measurements of serum lipids and lipoprotein catabolism in vivo; and

(iv) there were good experimental models (rabbit and mouse) for the human disease. Since the early 90s, both ex vivo and in vivo strategies have been designed. The first ex vivo protocol was designed to endow FH patients with a functional gene encoding LDL receptor, irrespective of the type of mutation causing the disease. In experimental animal studies based on an ex vivo approach, autologous hepatocytes were genetically corrected with recombinant retroviruses, and were subsequently transplanted back into the liver via the portal circulation.<sup>9</sup> The experiment resulted in a significant decrease in serum cholesterol levels. An early study on a first patient reported a significant decrease in serum LDL concentration (17%); these values remained stable for 18 months.<sup>10</sup> One year later, 5 patients with homozygous FH were enrolled in a pilot clinical trial: a large proportion (20% to 35%) of their livers was surgically removed, and normal LDL receptor genes were transferred into liver cell suspensions which were reinfused into the patients' livers via the portal vein. Significant and prolonged (at least 4 months) reduction in LDL cholesterol was demonstrated in 3 of the 5 patients.<sup>7,11</sup>

Other gene therapy strategies in FH patients were designed on the basis of in vivo adenoviral vectors.<sup>12,13</sup> The extraordinarily efficient uptake of adenovirus by liver cells, the availability of the portal vein for the injection of virus or of engineered cells, the likelihood that relatively broad ranges of gene expression would be tolerated, made FH an especially propitious candidate for adenovirus-mediated gene therapy. Experimental studies were performed in which mice were inoculated intravenously with recombinant adenovirus encoding

human LDL receptor, driven by the cytomegalovirus promoter or by the enzyme firefly luciferase as a reporter protein. Under these conditions, more than 99% of virus-dependent luciferase expression was detected in the liver (where cholesterol catabolism occurs). Four days later, levels of LDL receptor in liver had decreased tenfold, and the human receptor was detected in roughly 90% of liver cells.<sup>12</sup> In mutant mice lacking LDL receptor, injection with the virus of gene encoding LDL receptor restored the expression of receptor to the liver, accelerated the clearance of VLDL (which was impaired thirtyfold, even more than was the case with LDL), and corrected the lipoprotein profile of the hypercholesterolemic receptor-deficient mice.<sup>13</sup>

Both ex vivo and in vivo somatic gene therapy approaches are therefore applicable to FH. Promising but as yet nonoptimal results have been provided by both strategies.<sup>7,9-14</sup> It must be pointed out that, since the first description of FH, dietary restriction and treatments with lipid-lowering drugs have been widely employed with the aim of protecting coronary arteries and preventing coronary artery disease; interesting results have been obtained both with diet and with drugs, especially in heterozygotes.<sup>15</sup> Nevertheless, thanks to the dramatic evolution of human gene therapy and to the lack of efficiency of conventional treatments for the potentially fatal homozygous forms, gene therapy will likely be widely accepted and used.

• Another genetically determined form of dyslipidemia potentially suitable for gene therapy strategies is **familial dysbetalipoproteinemia**, in which a circulating abnormal lipoprotein,  $\beta$ VLDL (very-low-density lipoproteins), forms from



chylomicrons and VLDL remnants. The disease is a homozygous condition caused by the E2 isoform of apolipoprotein E (apo E).<sup>8,16</sup> Apo E is a major protein constituent of two of the classes of plasma lipoprotein involved in lipid transport and metabolism. It is synthesized in many different tissues, but liver is its predominant source. Apo E is a key regulator of cholesterol-rich lipoprotein metabolism: it is responsible for the uptake of chylomicrons and VLDL remnants because it contains a binding site that is complementary both to the remnant and to the LDL receptors. On cell surfaces, apo E therefore binds LDLRs, which are responsible for cholesterol uptake by the cell. Polymorphic variants of apo E have been shown to be associated both with elevated cholesterol levels and with an increased risk of early coronary artery disease. In humans, there are three apo E isoforms: E2, E3, E4 (genotypes  $\epsilon 2$ ,  $\epsilon 3$ ,  $\epsilon 4$ ).<sup>8</sup> Of the three, the E2 isoform shows the lowest binding affinity to hepatic receptors.<sup>17</sup> In familial dysbetalipoproteinemia, this low-binding affinity E2 isoform is the only one present; the absence of the strong binding isoforms of apo E results in increases in the half-life of the remnants in the serum. This remnant half-life allows lipoprotein lipase (in the capillary endothelium of adipous tissue and of skeletal muscle) and hepatic lipase to continue to act, and thus to reduce VLDL to the smaller  $\beta$ VLDL. Familial dysbetalipoproteinemia represents an essential precondition for type III hyperlipidemia, which develops in about 5% of patients affected by dysbetalipoproteinemia, and which is strongly associated with premature coronary artery disease.<sup>18,19</sup> The development of type III hyperlipoproteinemia depends on the addition or not of other genetic and/or environmental

factors. Genetic factors comprise: (i) genes responsible for familial combined hyperlipidemia + E2 homozygosity<sup>20</sup>; and (ii) hypothyroidism and diabetes. Environmental factors include sex, age, obesity, alcoholism, and nicotine.

Apo E-deficient mice develop marked hyperlipidemia as well as atherosclerosis, and represent good models for the evaluation of the effectiveness of gene therapy in human genetic dysbetalipoproteinemias. Recombinant adenovirus infusions containing either human apo E (vAdv.apo E) or the reporter gene luciferase (vAdv.luc) were administered intravenously to apo E-deficient mice with very high preinfusion plasma cholesterol levels. A single vAdv.apo E infusion led to the appearance of apo E in the plasma, to the normalization of lipid and lipoprotein profiles, and hence to a marked decrease in total cholesterol, a decrease in VLDL, IDL (intermediate-density lipoproteins) and LDL, and to an increase in HDL (high-density lipoproteins). The aortic atherosclerotic plaque area was significantly reduced with respect to controls.<sup>21</sup> The combined use of adenovirus vectors in the apo E-deficient mouse therefore represents a promising *in vivo* gene therapy approach.

Given that apo E is synthesized by numerous tissues/cells, including macrophages, other strategies are also progressing: it has recently been shown that atherosclerosis can be prevented in apolipoprotein E-deficient mice by bone marrow transplantation, thanks to apo E synthesis by normal macrophages from transplanted marrow.<sup>22</sup> Boisvert et al<sup>23</sup> performed bone marrow transplantation on hypercholesterolemic apo E-deficient mice with either syngeneic apo E-deficient mouse bone marrow cells (control) or wild-type mouse bone marrow cells expressing apo

E (treated). Both control and treated mice were fed either a regular chow diet or an atherogenic diet. Serum cholesterol levels dropped dramatically in the treated mice due to a reduction in their VLDL cholesterol. No changes were seen in the control group. Serum cholesterol (after 4 weeks) and the extent of atherosclerosis (after 14 to 16 weeks) were greatly reduced in the treated mice. Wild-type apo E mRNA was detected in the liver, spleen, and brain of the treated mice, documenting the successful apo E gene transfer, and the level of apo E expression was sufficient to reduce the hypercholesterolemia of the apo E-deficient mice fed either chow or atherogenic diets.<sup>23</sup>

- A further condition associated with increased cholesterol levels and therefore with increased risk for atherosclerosis is the **raised level of "lipoprotein small a" [(Lp(a))]** which is formed by the disulfide linkage of apolipoprotein B100 (apo B) of a LDL particle to apolipoprotein(a) [(apo(a))].<sup>24</sup> Apo B is the principal apolipoprotein constituent of LDL, which is the major lipoprotein involved in cholesterol transport. Apo A bears a striking resemblance to the fibrinolytic enzyme precursor plasminogen, and the genes for the two proteins are closely linked on chromosome 6.<sup>8</sup> Lp(a) is highly polymorphic, and its levels show up to 1000-fold variability in the population. Plasma levels of Lp(a) are almost entirely genetically determined: approximately 90% of Lp(a) variability can be attributed to the apo A gene.<sup>25</sup> Size polymorphism accounts for nearly 70% of Lp(a) variance, but even apo A alleles of the same size are heterogeneous at the DNA sequence level. High Lp(a) levels are associated with increased plasma cholesterol levels and with an increased risk of early coronary artery disease. Lp(a) may participate

both in thrombogenic and in atherogenic processes because of the plasminogen-like properties of apo A, and is possibly involved in the link between atherosclerosis and thrombosis.<sup>26</sup> There are no current strategies for Lp(a) gene therapy; however, given that conventional treatments fail to normalize Lp(a) and that current intervention focuses on the reduction of coexisting risk factors, apo A gene is a possible candidate for future investigation.

- A different strategy for atherosclerosis gene therapy aims at **protecting against, or at reversing, the disease; it is based on raising the levels of circulating HDL cholesterol.** In vivo strategies in mice by intravenous injection of recombinant adenovirus encoding human apolipoprotein AI (apo AI) resulted in an overexpression of apo AI; as a consequence, HDL cholesterol increased to levels that are known to be protective in humans.<sup>27</sup> Like other secreted circulating proteins, apo AI is suitable for gene therapy, since virtually any somatic cell could be targeted as a source of the given product. The expression of the gene declines rapidly, falling to <10% of peak levels within 12 days of injection. Although transient expression of a foreign gene may be sufficient to achieve specific goals for some applications, for chronic diseases, such as hypercholesterolemia, strategies for stabilizing therapeutic gene expression are critical to long-term efficacy after adenovirus-mediated gene transfer.

## HYPERTENSION

The polygenic nature of clinical hypertension has limited the identification of hypertension genes in humans. Nevertheless, genetic analysis in animal models and in human populations supports

the hypothesis that the renin-angiotensin system (RAS) plays an important role in hypertensive phenotype. Renin is an aspartic protease that cleaves angiotensinogen into the decapeptide angiotensin I; this cleavage is the rate-limiting step in the renin-angiotensin system.

Subsequent cleavage by angiotensin-converting enzyme (ACE) produces the octapeptide angiotensin II (AGII), which regulates blood pressure and salt retention. Molecular variants of angiotensinogen have been associated with an inherited predisposition to essential hypertension.<sup>28</sup> Molecular variants of the ACE genes have been associated both with ischemic heart disease and with atherosclerosis.<sup>29,30</sup> Molecular variants in the angiotensin II type I receptor gene, combined with the ACE gene variants, have been reported to synergistically increase the risk of myocardial infarction.<sup>31,32</sup> Trials in which ACE inhibitors have been found to decrease the occurrence of cardiovascular events [SAVE (Survival And Ventricular Enlargement Trial), SOLVD (Studies Of Left Ventricular Dysfunction)]<sup>33,34</sup> provide further confirmation of an association between the renin-angiotensin system and cardiovascular diseases, both via hypertension and directly. Although in vivo gene transfer of ACE has been performed in experimental animals,<sup>35</sup> no true gene therapy strategies have been attempted, since the gene defects in the RAS system are not fully elucidated, and since current knowledge suggests that the increase in risk could be associated with the given combined polymorphisms rather than with a single gene defect. An alternative association could be with dominant defects in one rather than in another gene of the RAS. In other words,

genetic polymorphisms that are biochemically and pathophysiologically related to a disease may represent the genetic background which makes certain individuals "more likely" to be affected.

Analysis of these polymorphisms may identify a group of markers whose combined effect significantly contributes to disease susceptibility. Since population studies can be undertaken more easily than family studies, especially for coronary artery disease, much work has been carried out with this approach. These studies are based on the rationale of identifying mutations in given genes that predispose to coronary artery disease by showing modifications in the frequency of a DNA variant (RFLP [restriction fragment length polymorphism], VNTR [variable number tandem repeat], DNA haplotype) cosegregating with the phenotype (ie, ACE gene D allele controlling >50% serum and tissue ACE levels and associated with increased risk for coronary atherosclerosis). A gene therapy strategy for such genetic risk markers is unlikely to be addressed in the near future. However, genetically guided drug therapy (eg, ACE inhibitors in patients with DD ACE genotype) appears to be feasible and logical, once the association between the risk phenotypes and coronary artery disease has been definitively assessed.

## HYPERHOMOCYSTEINEMIA

A further risk factor candidate for future gene therapy studies is hyperhomocystinemia. Severe forms, as in classic homocystinuria due to cystathionine synthase deficiency, which is inherited as an autosomal recessive trait, may cause premature atherosclerosis and thromboembolism. Recently, even mildly increased homocysteine levels



have been recognized as a serious risk factor in the development of atherosclerotic disease and thromboembolism.<sup>36</sup> Hyperhomocystinemia is associated both with deficiencies in the activity of one of several enzymes in methionine metabolism, notably the above mentioned cystathionine  $\beta$ -synthase, 5,10-methylenetetrahydrofolate reductase, and with disorders of methionine synthase activity due to a defect in methylcobalamin synthesis. Mild hyperhomocysteinemia can be the consequence of intermediate deficiency (about 50% remaining activity) of cystathionine synthase, or of the thermolability of a homozygous variant of 5,10-methylenetetrahydrofolate reductase. Variations in individual plasma homocysteine levels may also be caused by environmental factors or systemic diseases. These factors and diseases include: (i) deficiency of folate and vitamin B<sub>6</sub>; (ii) the antifolate drug methotrexate; (iii) antiepileptics; (iv) nitric oxide; (v) renal failure (the increase is correlated with serum creatinine); (vi) chronic liver disease; and (vii) type I diabetes mellitus. In 1976, Wilken and Wilken first published results suggesting that mild hyperhomocysteinemia played a role in the pathogenesis of coronary artery disease<sup>37</sup>: about 30% of young patients with angiographically proven coronary artery disease demonstrated mild hyperhomocysteinemia 4 hours after a methionine load; in 1992, pooled data revealed a prevalence of 32% in patients with peripheral vascular disease, of 24% in those with cerebrovascular disease, and of 21% in those with coronary artery disease.<sup>38</sup> It has also recently been shown that mild hyperhomocysteinemia is a strong risk factor for recurrent venous thrombosis, and that the condition can lead to a two- or threefold

increase in risk.<sup>39</sup> A possible relationship between plasma levels of homocysteine and conventional risk factors for vascular disease has been suggested, but no such relationship was established for tobacco smoking, hypertension, serum lipids, or diabetes mellitus. Hyperhomocysteinemia therefore seems to be an independent risk factor for coronary artery disease.<sup>36</sup> Although simple and inexpensive treatment based on vitamin administration and diet control can normalize homocysteine metabolism, it is not known whether such treatment will also reduce morbidity and mortality. Among emerging risk factors for atherosclerosis, hyperhomocysteinemia is an interesting candidate for future genetic studies and related therapeutic strategies, again on condition that the associated risk has been definitively assessed.

#### **“LOCAL” GENE THERAPY STRATEGIES AND ATHEROSCLEROSIS**

Given the systemic nature of atherosclerosis, gene therapy strategies designed for catheter-based local delivery devices of the therapeutic gene do not appear to be appropriate candidates for future application; a theoretical application would be morphologically risky plaques in ischemic heart disease patients, but we lack reliable markers, as well as certainty about the evolution of “at risk” lesions. Recent studies on animal models have focused on the prevention of restenosis after angioplasty by means of percutaneous delivery of an adenoviral vector.<sup>40</sup> Although the expression of the gene is transient in all experimental models, the temporary activity that characterizes the early postangioplastic period may possibly provide efficient prevention of smooth

muscle cell migration and proliferation, which have been shown to occur early after injury, and which are held responsible for restenosis.<sup>41,42</sup> The genetic material currently under consideration for gene transfer in postangioplasty atherosclerotic plaques consists of antisense oligonucleotides, which are used to inhibit the expression of oncogenes influencing cell proliferation (such as *c-myb* and *c-myc*).<sup>41</sup> Other approaches could be to target specific growth factor–signaling pathways, with local delivery of genes encoding receptor antagonists or soluble growth factor–binding proteins.<sup>40</sup> However, experimental studies are still needed, in particular to demonstrate the absence of potentially dangerous side effects due to the virus, and to increase the low level of transferred gene expression in the target tissue. Once effective local delivery systems and gene therapy strategies are available, it will be theoretically possible to move from “ideal lesions,” such as postangioplasty restenosis, to spontaneous atherosclerotic plaques, especially those characterized, for example, by neointimal smooth muscle cell proliferation similar to that of restenosis, such as have been demonstrated in a high percentage of culprit lesions in unstable angina, stable angina, and sudden coronary death patients.

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