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Molecular Alterations Critical to the Development of Arteriosclerotic Plaques: A Role for Environmental Agents

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Cardiovascular disease (CVD) is the single greatest cause of death in the United States and in Western Europe. There is strong epidemiologic evidence for an interaction of environmental and genetic factors in the development of clinically significant episodes of CVD. However, the specific contributions of each of these components to the onset and development of CVD remain unclear. According to the monoclonal hypothesis, arteriosclerotic plaques, the principal lesions associated with CVD, are monoclonal in origin and can be considered benign smooth muscle cells tumors of the artery wall. It follows that somatic cell alterations, possibly brought about by chemical mutagens or viruses, may play critical roles in plaque formation.

During the past decade, evidence has been presented from a number of laboratories, including ours, that in animal model systems, both viruses and chemical carcinogens can play a key role in the appearance and development of arteriosclerotic plaques. We have recently provided evidence consistent with the view that somatic cell alterations are critical to plaque development in man: DNA from human arteriosclerotic plaques transforms cells *in vitro* and injection of these transformed cells into nude mice results in tumor formation. Thus, plaque DNA behaves similarly to tumor DNA under defined assay conditions.

Introduction

During the past decade, there have been profound shifts in the types of questions asked and the experimental approaches adopted by investigators in the field of environmental health. In part, this is due to the growing recognition that a very large number of exogenous agents acting singly, together, or synergistically can have profound effects upon the etiology and/or development of a wider variety of diseases and illnesses than had previously thought to be the case. This is also partly due to tremendous technical advances. Analytical instrumentation and methodologies have progressed to the point that the molecular mechanisms that underlie the responses of cells, tissues, and organisms to environmental stressors can now be investigated. In the discussion that follows, recent findings will be considered that implicate environmental agents in the etiology of arteriosclerotic plaques. Additionally, evidence will be summarized that similar molecular mechanisms may underlie critical events in the development of plaques and tumors.

In cancer studies, it has been well accepted for many years that at least two distinct cellular events (hits) are necessary to effect cell transformation *in vitro* or carcinogenesis *in vivo*. If one of these events is derived endogenously (a germ line mutation or a spontaneous somatic mutation are two possibilities), then an environmental insult resulting in a second complementary mutation (e.g., activation of the *ras* oncogene) may be sufficient to trigger transformation. The role of environmental agents in oncogene activation is considered by other contributors to this volume.

Although there is much discussion in this volume of cancer and the role of environmental agents in its development, the leading cause of death in most Western countries, including the United States, is not cancer, but rather, cardiovascular disease (CVD). Environmental factors (e.g., cigarette smoke and agents that elevate blood pressure) have been associated with the development of clinically significant episodes of CVD. However, evidence linking these factors to CVD in a causal way, let alone the demonstration that these factors are directly involved in the molecular events which underlie development of CVD, are lacking.

The principal lesion characteristic of CVD is the arteriosclerotic plaque. The plaque consists of cellular elements [primarily smooth muscle cells (SMC) but also some macrophages] and formed elements (including lipid deposits, collagen, elastin, and glycosaminoglycans). In advanced plaques, necrosis and calcification may occur. Critical to the development of plaques is the proliferation of SMC within the intimal region of the artery wall.

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Results and Discussion

Injury versus Monoclonality

The traditional view is that the stimulus to SMC proliferation is injury (*1*), e.g., by hemodynamic factors or bloodborne agents. Although there is considerable circumstantial evidence to support the contention that these factors play a role in plaque formation, the evidence is less compelling that these factors are the primary causes of arterial SMC proliferation *in vivo* and the subsequent plaque development. If the plaques arise as a response to injury, then the plaques should be polyclonal as are other healing wounds. That is, the cells that occupy the wound site and repopulate it during the healing process are derived from many different cells, even if only one cell type is involved.

An alternative to the injury-plaque scenario was provided by isozyme studies on plaques obtained from autopsy samples. Evidence was obtained that human arteriosclerotic plaques are monoclonal in origin (*2*). An argument can be made that apparent monoclonality could arise because of strong selection pressure favoring a subpopulation of cells that appear in response to wound healing. However, a simpler, more straightforward, albeit controversial, conclusion presents itself, namely, the monoclonality is evidence that the plaques are benign SMC tumors of the artery wall. The monoclonal hypothesis, as it came to be known, provided new insight into the study of plaque etiology and in the process established a role for environmental medicine in these investigations. Environmental agents that had been implicated previously in cell transformation and tumorigenesis now became candidates for involvement in arteriosclerosis as well. An early prediction that arose from the monoclonal hypothesis was that viruses and chemical mutagens would be expected to play critical roles in plaque formation and development, just as they do in tumorigenesis.

Viruses and Mutagens in Plaque Development

The earliest attempt at verifying this prediction came from Roy Albert and his co-workers at NYU's Department of Environmental Medicine. Weekly injections of the polycyclic aromatic hydrocarbon (PAH) carcinogens 7,12-dimethylbenz[a]anthracene (DMBA) or benzo[a]pyrene (BaP) resulted in large, proliferating plaques in the abdominal aorta in cockerels (*3*). Plaques increased in size in a dose-dependent fashion (*4*), and even a single injection at 5 weeks of age was sufficient to cause a marked increase in thymidine-labeling index of plaque SMC (*5*) within 1 week. Enzymes of the cytochrome P-450 system have been identified in both the adult artery wall (*6,7*) and in fetal aortic SMC (*8*). The analogy between plaques and benign tumors was strengthened by a recent demonstration that DMBA and methoxamine, administered in an initiation-promotion protocol, resulted in the appearance of microscopic, focal thoracic aorta plaques in cockerels (*9*).

The PAH effect is more complex than it was originally thought to be on at least two accounts. First, some noncarcinogenic PAHs also stimulate plaque development (Penn, manuscript in preparation). Second, extensive temporal studies (*5*), as well as light and electron microscopic (*10*) investigations, revealed that rather than causing new plaques to appear, the principal effect of PAH administration, at least in cockerels, is to accelerate the development of preexisting aortic plaques. For example, aortic plaques in 8-week-old cockerels that received four weekly injections of DMBA are the same size as plaques in 20-week-old control animals (*11*).

The second prediction arising from the monoclonal hypothesis, that viruses might play a critical role in plaque formation, was borne out as well. Single injections of the oncogenic avian herpes virus, Marek's disease virus, into 4-day-old cockerels yielded focal microscopic plaques in the thoracic aorta (*12,13*). A possible link was recently inferred between viral infection and alterations in lipid metabolism, the latter being more traditionally associated than the former with the development of CVD. Aortas of cockerels infected *in vivo* with Marek's disease virus and arterial SMC infected *in vitro* with this virus both displayed altered synthesis and accumulation of cholesterol esters (*14,15*). There is one report that herpes virus genomes have been identified in human artery wall and possibly even in plaques (*16*). However, no strong evidence yet exists that viral infection is an etiologic agent in plaque formation in man.

The fact that viruses and chemical carcinogens can play a key role in experimental arteriosclerosis is interesting, but by itself ultimately unsatisfying. From a medical perspective, it is vital to show that these agents can play a role in development of clinically significant arteriosclerotic plaques. Just as importantly would be the demonstration of a viable molecular mechanism to explain how viruses and mutagens could play a critical and possibly irreversible role in plaque formation. This would also help to confirm the putative relationship between plaques and tumors.

Somatic Cell Alterations and Plaque Development

Molecular biology techniques developed over the past decade have permitted direct demonstration that specific types of genetic alteration are characteristic of cell transformation and tumor development. In our laboratory, we have adapted these procedures to studies of human plaques. Specifically, we have demonstrated via gene transfer procedures that human plaque DNA is capable of transforming NIH 3T3 cells in culture (*17*). Following injection into nude mice, these transformants consistently give rise to panels of slowly growing tumors. The identity of the plaque-derived transforming gene(s) is not yet known. It is important to recognize that the NIH 3T3 cell gene transfer assay that we have employed is most effective at identifying a limited range of dominant transforming genes. Recent evidence has demonstrated that as other assays of increasing sensitivity are developed, the

chances increase of more readily identifying transforming genes (*18,19*). We are currently investigating the effectiveness of these assays with plaque DNAs of both human and animal origin.

A different approach to the question of genetic alterations in plaques or in cells that might become plaques has recently been described. Mildly elevated levels of c-*sis* mRNA have been found in human atherosclerotic plaques compared to the levels found in normal artery (*20*); c-*sis* is the cellular homologue of the *sis* oncogene. The gene product in both cases is essentially identical to the β-chain of the growth factor PDGF. The association of platelets with areas of arterial wall injury, the subsequent release of platelet factors, presumably including PDGF, and the ensuing proliferation of arterial wall SMC have been well documented in experimental systems. Whether similar events are relevant to clinically significant plaque development remains to be determined.

Prognosis

Elevated levels of serum cholesterol, high blood pressure, and cigarette smoking are the three leading independent risk factors for the development of CVD. Epidemiological evidence indicates that in an individual with two or more of these risk factors, the likelihood of suffering a CVD episode increases multiplicatively (*21*). However, no causal relationship has been identified between these factors and CVD. In addition, many people suffering from CVD (> 40%) display none of these risk factors. The possibility is strong that an interplay of environmental agents with genetic factors is necessary in many cases for CVD to manifest itself in a clinically meaningful way.

Carcinogenesis studies of the past few years have identified specific environmental agents (PAHs, alkylating agents, X-rays, even O_3) that are capable of activating transforming genes. In some cases, the mechanism of action (e.g., induction of a mutation in codon 12 or codon 61 of the *ras* gene) has been identified. Once the transforming gene(s) present in arteriosclerotic plaques is identified, it will become feasible to ask how specific environmental agents interact with this gene to activate it.

The existence of an inexpensive animal model exhibiting rapid plaque development, the cockerel, allows us to ask at what point during or prior to plaque development is the transforming gene activated. The human samples studies so far are from middle-aged to elderly patients with severe CVD. Thus, it is impossible to correlate activation of a transforming gene with a stage of plaque development in these samples.

Studies of human fatty streaks, a precursor lesion to plaques, indicate that there are subpopulations with monoclonal characteristics (*22*). Fatty streaks have also been identified in animal model systems, including monkeys, pigs, and rabbits. Results from our laboratory summarized earlier indicate that in cockerels, the effect of carcinogens is to accelerate plaque development. The monoclonal fatty steaks may represent a population of already-mutated cells that require a second mutagenic event, e.g., by an environmental agent, in order to develop into a fully developed plaque. This can be tested both *in vitro* and *in vivo*.

Finally, the tumors that arise in mice following injection of plaque DNA-transformed cells develop much more slowly than do most tumors arising following injection of cells containing activated oncogenes (7–16 weeks for plaque DNA associated tumors versus < 2 weeks for activated *ras*-associated tumors). As yet, no reasonable explanation has been presented for why in plaque-associated tumors there is such a long latent period, as well as an extended period of development once the tumors become visible. These questions are especially interesting because there are no other clear-cut differences between plaque-associated tumors and *ras*-associated tumors.

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