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Commentary

Death by Any Other Name

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This issue of the Journal contains two papers, one by Han and collaborators[42] and one by Geng and Libby,[43] describing apoptosis in the atherosclerotic plaque. The authors of these articles have detected cell death by using assays for DNA fragmentation, staining of nuclear and extranuclear DNA, and morphology of cells. They describe the appearance of the dead cells as apoptosis, a term we have also recently used to describe cell death in cultured human plaque smooth muscle cells.[1] Why is this information important?

At the simplest level, the implication is that apoptosis is a distinct form of cell death, distinguishable from classical necrosis. However, the terminology has become confusing, and apoptosis may mean different things to different users of the term.

As used by pathologists,[2,3] necrosis is a general term referring to morphological stigmata seen after a cell has passed the point of no return, that is, after a cell has committed to die. The point at which cells are irreversibly injured is difficult to define; instead, we define cell death by later events, morphological changes called necrosis. The presence of necrosis tells us that the cell has died and the type of necrosis sometimes tells us about how the cell has died. Used in this way, apoptosis is one form of necrosis.

The characteristic morphological changes of apoptotic death have been well described recently in an editorial in this journal by Majno and Joris.[2] Apoptotic death occurs by shrinkage of the cell, a process that Majno and Joris distinguish from cell swelling, or "oncosis," seen in some other forms of necrosis. Moreover, death by apoptosis is characterized by the maintenance of membrane integrity over most of the process, and apoptotic bodies are generally rapidly phagocytosed by adjacent cells. Thus, this form of cell death usually does not result in release of intracellular contents and the ensuing inflammatory response that such release provokes.[4]

As recently reviewed in Science,[5–7] apoptosis is also used as a synonym for programmed cell death. Programmed cell death is a physiological form of cell death required for normal metazoan development or function, as seen, for example, during embryogenesis or in amplification of immunogenic T cell clones. This form of death characteristically involves utilization of a series of proteins whose genes have been mapped by classical mutation analyses or more modern methods of genetic manipulation. Other apoptotic or anti-apoptotic genes have been identified in viral genomes.[5–7] Although many of the details of the genetic pathways leading to programmed cell death have been elucidated (Figure 1), it is essential to realize that the morphological pattern of apoptosis does not itself tell us that any specific mechanism has caused cell death. Indeed, the morphological pattern of apoptosis can occur in response to a wide range of stimuli, including the genetically defined pathways described below, as well as such classical examples of cell death as myocardial ischemia[4,7–10] or central nervous system ischemia.[7] Of more importance to us, then, is whether the use of the word apoptosis provides new information about the ontogeny of atherosclerosis. For example, does the presence of apoptotic cells imply a specific process of cell death somehow indicative of a process likely to be important in plaque progression?

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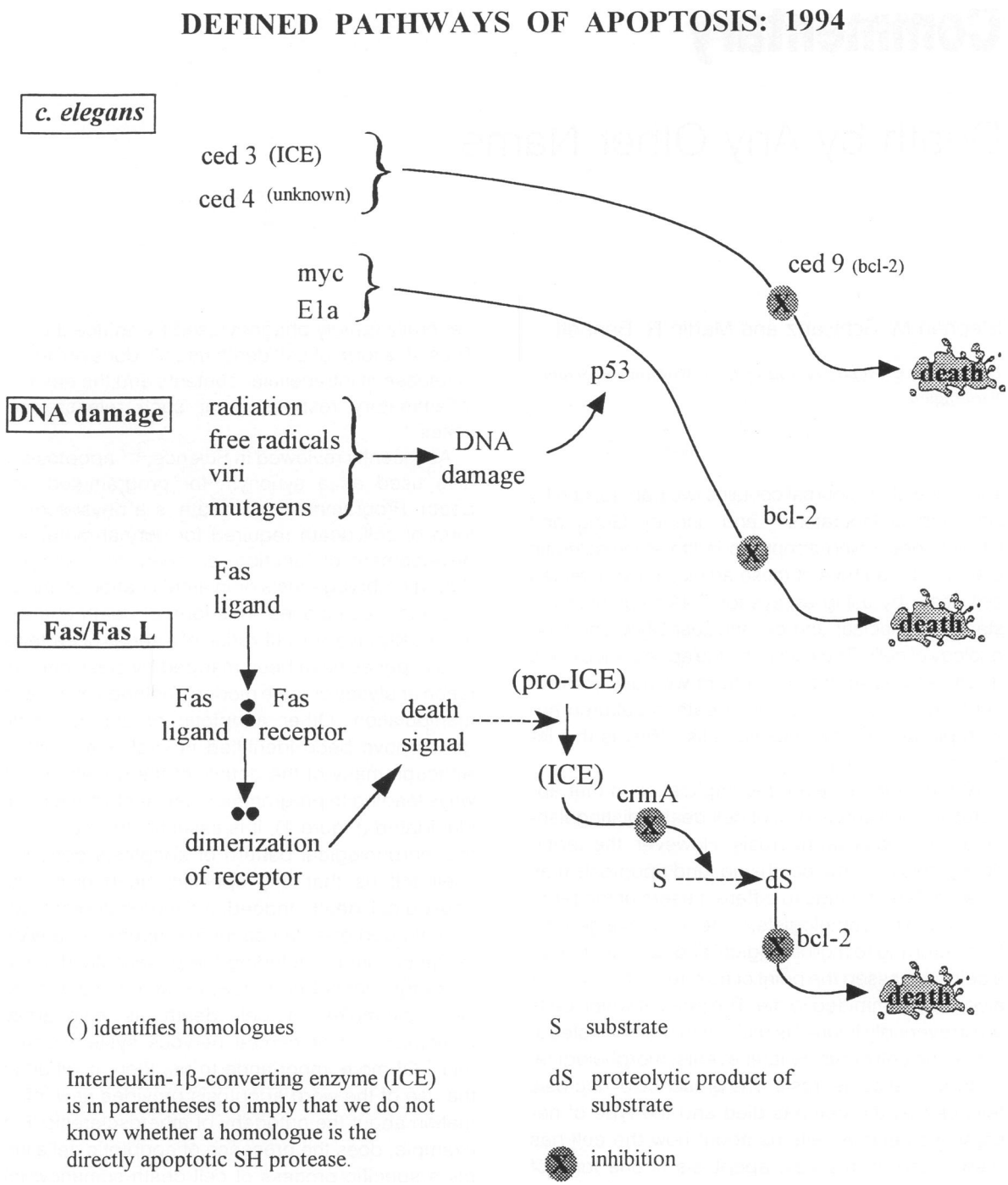

Figure 1. *Pathways of cell death. Genetic studies in* C. elegans *as well as spontaneous mutations in higher organisms have led to identification of specific pathways controlling programmed cell death.*

Observations of cell death, and even the suggestion of programmed cell death in atherosclerosis, are not themselves new. In his lectures of 1858, Virchow described the process of atherosclerosis as depending upon first the replication of cells in the plaque and then the death of these same cells. The critical sentence is eerily reminiscent of modern concepts of apoptosis: "Thus, we have here an active process which really produces new tissues, but then hurries on to destruction in consequence of its own development."[11] Virchow thus identifies cells that appear destined, or are programmed, to die. Reports of cell death in plaques since that time have been many. Perhaps

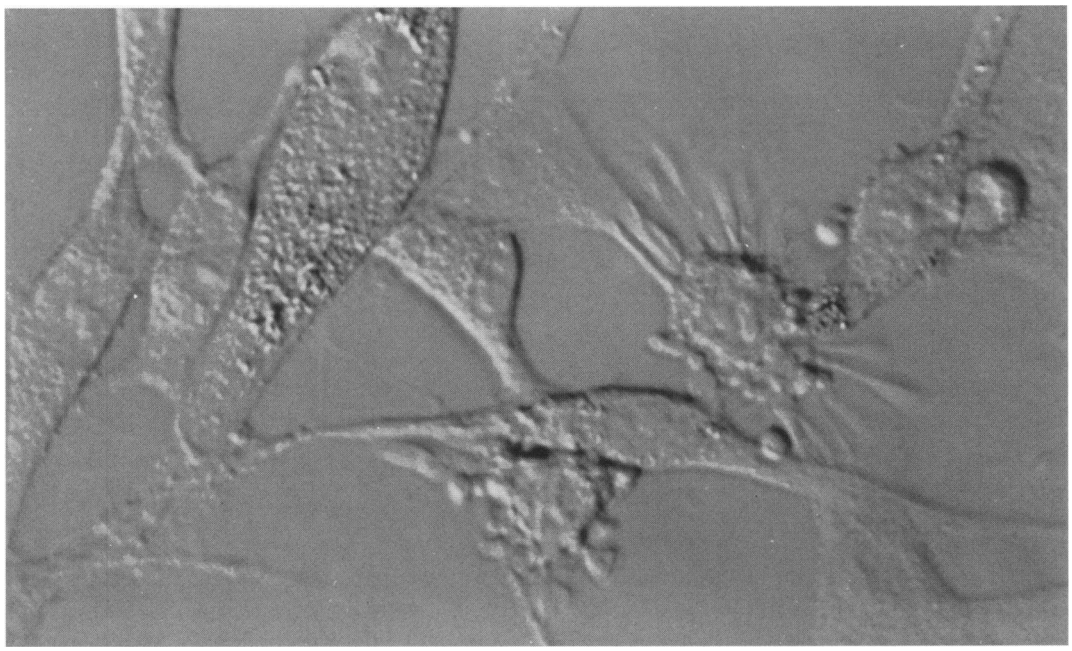

Figure 2. *Human plaque smooth muscle cells. Time-lapse video studies show that cultured human plaque smooth muscle cells have a high spontaneous rate of cell death. These cells progress rapidly through characteristic changes with breakup of the cell into apoptotic bodies and uptake of these apoptotic bodies by adjacent cells.*

the most relevant are quantitative studies by Thomas et al[12,13] in fat-fed animals. These workers used elegant cell kinetic modeling studies to associate cell death in atherosclerotic plaques with the presence of oxidized products in the cholesterol diet, a very contemporary idea today.[14] Moreover, Thomas and his collaborators suggested that cell death was linked to cell replication, an idea that is central to a current concept of one mechanism of apoptosis (below). Thus, neither the observation of cell death in atherosclerotic plaques, nor the concept of programmed cell death, is new.

It is possible that these articles offer us new information about the rate of cell death in advanced plaques. Both studies demonstrate surprisingly high percentages of cells positive for the terminal transferase (TUNEL) reaction of up to 40%. Unfortunately, estimates of rates of cell death are difficult to derive from the frequency of TUNEL-positive cells, as we have no convincing way to estimate the duration of time a cell undergoing apoptosis will display a positive reaction using terminal transferase to detect fragmented DNA. There is evidence, moreover, that cell death occurring by non-apoptotic mechanisms is also TUNEL-positive after 48 hours, when extensive DNA breaks have occurred.[8] Evidence from *in vitro* studies of human smooth muscle cells undergoing apoptosis[1] and from animal studies[15] suggests that apoptosis is a rapid process, lasting 2 to 4 hours. However, if, as Geng and Libby[43] suggest in this issue, the apoptotic bodies are not removed, or remain TUNEL-positive after engulfment, then high labeling indices may be independent of the time course of cell death itself.

The important point emerging from both of these studies, therefore, is not the rate of apoptosis, but the fact that it is occurring. Moreover, there is evidence that plaque smooth muscle cells may well have a form of programmed cell death. We have recently demonstrated higher rates of apoptosis in cultured smooth muscle cells from human plaques than cells from normal vessels[1] (Figure 2).

The increased susceptibility to apoptosis in plaque-derived cells, and the fact that this property persists in culture, suggests that apoptosis in plaque-derived smooth muscle cells is due to an intrinsic property of the cells, ie, to programmed cell death. There are now several examples of specific genetically determined apoptotic pathways. The most compelling example of such a cell death pathway comes from studies of the nematode *Caenorhabditis elegans*. Fate maps in *C. elegans* identify cells destined to die during development. Genes regulating this cell death have been identified and, by genetic complementation, the beginnings of a pathway can be discerned. The critical elements of this pathway so far are two pro-

apoptotic genes, *ced*-3 and *ced*-4, and one anti-apoptotic gene, *ced*-9. These genes in *C. elegans* have structural and functional homology with genes expressed in our own species. The *C. elegans* gene *ced*-9 protects against apoptosis and appears to be homologous to the proto-oncogene *bcl*-2.[16,17] Failure of both *ced*-9 and *bcl*-2 to be turned off at the required point results in excess cells surviving, which, in the case of *bcl*-2, results in follicular lymphoma.[18] As already noted, the human plaque smooth muscle cell has a high rate of apoptosis *in vitro*. We have also recently shown that *bcl*-2 is able to protect plaque-derived smooth muscle cells against apoptosis *in vitro*.[1] Although *bcl*-2 itself was not found in smooth muscle cells in our study, *bcl*-2 is one member of a family of often-interacting proteins whose expression or loss of expression could be responsible for accelerated apoptosis in cells of the atherosclerotic plaque.[18] *ced*-3 also appears homologous to a mammalian gene, the interleukin-1β converting enzyme (ICE).[19] We know that *ced*-3 promotes apoptosis; we also know from transfection studies that high levels of ICE expression can induce apoptosis in mammalian cells.[19] However, we lack genetic evidence proving that ICE, and not some homologous gene, induces apoptosis under physiological or pathological conditions in mammalian cells. (Indeed, evidence from ICE "knockout" animals suggests that ICE expression is not crucial to apoptosis occurring during embryogenesis or in early postnatal development (Science 1995, 267:2000).)

It is important to realize that the ICE/*bcl*-2 pathway outlined above appears to be independent of cell proliferation. Thus, *bcl*-2 will prevent apoptosis without affecting replication of the cell, even when proliferation is promoted by the same gene product as that inducing apoptosis.[20,21] In contrast, we have identified c-*myc* and p53, genes important for controlling cell proliferation, as also regulating apoptosis in smooth muscle.[22] Our studies, as well as work in other cell types, indicates that the pathways mediating cell death and proliferation may be closely linked. For example, p53 is up-regulated after DNA damage induced by irradiation.[23,24] Frequently, up-regulation of p53 mediates a cell cycle arrest in G1. If DNA damage is extensive, however, p53 mediates apoptosis of that cell, preventing propagation of the damaged DNA. It is intriguing to note reports, so far unconfirmed, of overexpression of both c-*myc* or p53 in atherosclerotic lesions or sites of restenosis after angioplasty.[25,26] Although these studies suggested that overexpression of the gene in question was related to cell proliferation, advanced plaques or sites of restenosis show very little replication.[27,28] We have also demonstrated that, upon deprivation of growth factors, up to 20% of plaque-derived smooth muscle cells undergo apoptosis over 24 hours.[1] Thus, the major importance of findings of high levels of expression of specific genes, or the presence of growth factors in plaques,[29–32] may be the role these molecules play in determining whether cells in plaques survive rather than whether the cells proliferate.

A third source of information about the genetics of apoptosis comes from studies of viral proteins. Successful virus infection appears to depend in part upon suppression of apoptosis in the host cell. Apoptosis-inhibiting genes include adenovirus E1B^{55k} and the cowpox gene *crm*A, which suppress the activity of p53 and ICE-like proteases, respectively.[33,34] Other viral genes, such as E1B^{19k} or the baculovirus gene p35, also suppress host cell apoptosis.[34,35] As with the *C. elegans* data, the presence of anti-apoptotic genes in viruses suggests that these are natural pathways that function in both physiology and pathology, rather than an artifact of cell culture. The existence of *crm*A, for example, suggests that cells may have natural ICE-like enzymes that promote cell death after certain kinds of injury or infection.

A fourth class of genes associated with apoptotic cell death includes specific cell death receptors. By analogy to growth factor receptors, the existence of cell death receptors illustrates a central concept of apoptosis; that is, the receptor itself is not toxic to cells. The best studied of these receptors is Fas. Interaction of Fas with the Fas ligand, or cross-linking the Fas receptor with stimulatory antibodies, initiates a sequence of events that is probably finally mediated by intracellular ICE-like proteases.[6,36] Fas, a member of the tumor necrosis factor-α receptor family, signals cell death at appropriate times of expansion of T cell populations.[5–7] When this gene fails to be expressed, the resulting expansion of lymphocyte clones can produce a lupus-like immune state in transgenic mice, and perhaps even in humans. Thus, the Fas pathway is clear evidence that programmed cell death plays a functional role even in the adult organism. As we will discuss below, there are examples of physiological and pathological processes that likely involve programmed cell death pathways in the vessel wall.

Two specific components of plaque progression would seem to be good candidates for an apoptotic mechanism. The first of these is the dilatation of the vessel wall that occurs as atherosclerotic lesions progress. It is now well known that the atherosclerotic lesion can develop substantially without encroaching on the lumen.[37] This occurs be-

cause of a dilatation of the remaining vessel wall, a dilatation that is now frequently called remodeling. Remodeling, however, means that somehow the mass of the vessel wall and cell number have been redistributed. An obvious possibility is that remodeling depends upon the coordinated action of proliferation and cell death at various sites within the vessel wall. Direct evidence for such a mechanism comes from a recent report in newborn sheep vessels.[15] In vessels that undergo substantial changes in caliber after birth, the changes in DNA content of the vessel wall appear to be regulated by changes in the rate of apoptotic cell death, rather than cell proliferation. A second component of the lesion that would seem like a prime candidate for studies of apoptosis is the fibrous cap. Both studies of atherosclerotic plaque apoptosis appearing in this issue demonstrate apoptosis in macrophage-rich areas of the plaque. Macrophage-rich areas typically include shoulder regions where it has been suggested that rupture of the plaque occurs.[38–40] Apoptosis in this area may weaken the plaque and predispose it to rupture.

Finally, although much attention is focused on the smooth muscle cell, it is important to note once again that the atherosclerotic plaque is much more complex that just smooth muscle cells. Prominent cells in the plaque include macrophages, lymphocytes, and endothelial cells. All of these cell types undergo apoptosis *in vitro*. Indeed, whereas smooth muscle cells in advanced plaque show relatively little replication,[27] both endothelial cells and macrophages, especially focally, show significant replication. What we do not know is whether this replication represents an active form of growth of the plaque or cell turnover.

In summary, until now the focus of most interest in atherogenesis has been on how plaque cells grow.[41] With the articles in this issue and our own study demonstrating a higher apoptotic rate in cultured plaque-derived smooth muscle cells, we now need to include programmed cell death as an additional mechanism in our thinking about the pathogenesis of atherosclerosis and arterial remodeling.

References

1. Bennett MR, Evan GI, Schwartz SM: Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest 1995, 95:2266–2274
2. Majno G, Joris I: Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 1995, 146:3–15
3. Cotran RS, Kumar V, Robbins SL, Shoen FJ (Eds): Pathologic Basis of Disease, ed 5. Philadelphia, WB Saunders, 1994
4. James TN: Normal and abnormal consequences of apoptosis in the human heart: from postnatal morphogenesis to paroxysmal arrhythmias. Circulation 1994, 90:556–573
5. Steller H: Mechanisms and genes of cellular suicide. Science 1995, 267:1445–1449
6. Nagata S, Golstein P: The Fas death factor. Science 1995, 267:1449–1456
7. Thompson CB: Apoptosis in the pathogenesis and treatment of disease. Science 1995, 267:1456–1462
8. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassman H: Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation. Lab Invest 1994, 71:219–225
9. Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroc M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res 1994, 75:426–433
10. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994, 94:1621–1628
11. Virchow R: Cellular Pathology as Based Upon Physiological and Pathological Histology, ed 2. Birmingham, AL, Classics of Medicine Library, 1858, p 361
12. Thomas W, Kim D, Lee K, Reiner J, Schmee J: Population dynamics of arterial cells during atherogenesis. XIII. Mitogenic cytotoxic effects of a hyperlipidaemic (HL) diet on cells in advanced lesions in the abdominal aortas of swine fed an HL diet for 270–345 days. Exp Mol Pathol 1983, 39:257–270
13. Thomas WA, Reiner JM, Florentin FA, Lee KT, Lee WM: Population dynamics of arterial smooth muscle cells. V. Cell proliferation and cell death during initial three months in atherosclerotic lesions induced in swine by hypercholesterolemic diet and intimal trauma. Exp Mol Pathol 1976, 24:360–374
14. Steinberg D: Antioxidants in the prevention of human atherosclerosis: summary of the proceedings of a National Heart, Lung, and Blood Institute workshop. Circulation 1992, 85:2337–2344
15. Cho A, Courtman DW, Langille BL: Apoptosis (programmed cell death) in arteries of the neonatal lamb. Circ Res 1995, 76:168–175
16. Hengartner MO, Ellis RE, Horvitz HR: *Caenorhabditis elegans* gene *ced*-9 protects cells from programmed cell death. Nature 1992, 356:494
17. Hengartner MO, Horvitz HR: Activation of *C. elegans* cell death protein *ced*-9 by an amino-acid substitution in a domain conserved in *bcl*-2. Nature 1994, 369: 318–320
18. Hockenbery DM: The *bcl*-2 oncogene and apoptosis. Semin Immunol 1992, 4:413–420

19. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J: Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced*-3. Cell 1993, 75:653–660
20. Fanidi A, Harrington E, Evan G: Cooperative interaction between c-*myc* and *bcl*-2 proto-oncogenes. Nature 1992, 359:554–556
21. Rao L, Debbas M, Sabbatini P, Hockenberry D, Korsmeyer S, White E: The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and *bcl*-2 proteins. Proc Natl Acad Sci USA 1992, 89: 7742–7746
22. Bennett MR, Evan GI, Schwartz SM: Apoptosis of rat vascular smooth muscle cells is regulated by p53 dependent and independent pathways. Circ Res 1995 (in press)
23. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci USA 1992, 89: 7491–7495
24. el Diery WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994, 54:1169–1174
25. Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE: Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 1994, 265:391–394
26. Toda T, Tamamoto T, Sadi AM, Kiyuna M, Nakashima Y, Inoue Y: Expression of platelet-derived growth factor and c-*myc* in atherosclerotic lesions in cholesterol-fed chickens: immunohistochemical and *in situ* hybridization study. Virchows Arch 1994, 425:55–61
27. O'Brien ER, Alpers CE, Stewart DK, Ferguson M, Tran N, Gordon D, Benditt EP, Hinohara T, Simpson JB, Schwartz SM: Proliferation in primary and restenotic coronary atherectomy tissue: implications for antiproliferative therapy. Circ Res 1993, 73:223–231
28. Gordon D, Reidy MA, Benditt EP, Schwartz SM: Cell proliferation in human coronary arteries. Proc Natl Acad Sci USA 1990, 87:4600–4604
29. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H: Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 1990, 248:1009–1012
30. Majesky MW, Reidy MA, Bowen-Pope DF, Wilcox JN, Schwartz SM: Platelet-derived growth factor (PDGF) ligand and receptor gene expression during repair of arterial injury. J Cell Biol 1990, 111:2149–2158
31. Wilcox JN, Smith KM, Williams LT, Schwartz SM, Gordon D: Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by *in situ* hybridization. J Clin Invest 1988, 82:1134–1143
32. Rekhter MD, Gordon D: Does platelet-derived growth factor-A chain stimulate proliferation of arterial mesenchymal cells in human atherosclerotic plaques? Circ Res 1994, 75:410–417
33. Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ: Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1-β converting enzyme. Cell 1992, 69:597–604
34. Debbas M, White E: Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev 1993, 7:546–554
35. Clem RJ, Miller LK: Control of programmed cell death by the baculovirus genes p35 and *iap*. Mol Cell Biol 1994, 14:5212–5222
36. Tewari M, Dixit VM: Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus *crm*A gene product: J Biol Chem 1995, 270:3255–3260
37. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ: Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987, 316:1371–1375
38. Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J: Risk of thrombosis in human atherosclerotic extracellular lipid, macrophage, and smooth muscle cell content. Br Heart J 1993, 69:377–381
39. Henney AM, Wakeley PR, Davies MJ, Foster K, Hembry R, Murphy G, Humphries S: Localization of stromelysin gene expression in atherosclerotic plaques by *in situ* hybridization. Proc Natl Acad Sci USA 1991, 88:8154–8158
40. Arbustini E, Grasso M, Diegoli M, Pucci A, Bramerio M, Ardissino D, Angoli L, de Servi S, Bramucci E, Mussini A: Coronary atherosclerotic plaques with and without thrombus in ischemic heart syndromes: a morphologic, immunohistochemical, and biochemical study. Am J Cardiol 1991, 68:36B-50B
41. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993, 362:801–813
42. Han DKM, Haudenschild CC, Hong MK, Tinkle BT, Leon MB, Liau G: Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. Am J Pathol 1995, 147:267–277
43. Geng Y-J, Libby P: Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1β-converting enzyme. Am J Pathol 1995, 147:251–266