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Cell proliferation in human coronary arteries

(cyclin/smooth muscle/macrophage/atherosclerosis)

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ABSTRACT **Despite the lack of direct evidence for cell multiplication, proliferation of smooth muscle cells in human atherosclerotic lesions has been assumed to play a central role in ontogeny of the plaque. We used antibodies to cell cycle-related proteins on tissue sections of human arteries and coronary atherosclerotic plaques. Specific cell types were identified by immunochemical reagents for smooth muscle, monocyte–macrophages, and other blood cells. Low rates of smooth muscle cell proliferation were observed. Macrophages were also observed with rates of proliferation comparable to that of the smooth muscle. Additional replicating cells could not be defined as belonging to specific cell types with the reagents used in this study. These findings imply that smooth muscle replication in advanced plaques is indolent and raise the possibility of a role for proliferating leukocytes.**

Haust (1) first demonstrated the prominence of smooth muscle cells in human atherosclerotic plaques. Since then, smooth muscle proliferation has been assumed to be a critical part of the pathogenesis of atherosclerosis (2–4). This concept was supported by the demonstration that human plaques are monoclonal (5, 6), as was the idea that lesions originate by cell proliferation rather than by migration of polyclonal cells from the media (3, 4). Monoclonality, however, is indirect evidence for proliferation. While there is direct evidence for smooth muscle proliferation in animal models, direct, quantitative evidence of proliferation has not been obtained in human lesions. Moreover, the animal lesions appear to be polyclonal and could, therefore, arise by a different mechanism (7, 8). The recent development of monoclonal antibodies to proliferation-associated antigens allowed us to measure directly proliferation in human tissues (9–11). We used a monoclonal antibody to the proliferating cell nuclear antigen (PCNA; also known as cyclin). We first verified the specificity and sensitivity for proliferation in paraffin-embedded material by comparison with *in vivo* [^{3}H]thymidine labeling in rat tissues. The levels of PCNA reactivity were then determined in samples of normal and atherosclerotic human coronary arteries. Finally, by simultaneously using cell type-specific antibodies with a double immunolabeling technique, the cell types displaying proliferative activity were determined.

MATERIALS AND METHODS

Rat Tissue Preparation. Three-month-old Sprague–Dawley rats (Tyler Laboratories, Bellevue, WA) were studied 30 and 72 hr after aortic and left carotid balloon catheter injury (12). One hour before sacrifice, each animal was given a single intraperitoneal dose of [^{3}H]thymidine (0.5 μCi/g body weight; 6.7 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). The carotid arteries were perfused with lactated Ringer's solution and harvested, as were portions of small bowel and skin. All tissues were fixed overnight in methyl Carnoy's fixative [methanol/chloroform/glacial acetic acid, 60:30:10 (vol/vol)], then paraffin embedded, and sectioned at 6 μm thickness. After immunocytochemical staining (see below), thymidine autoradiography was performed (12). Staining of all nuclei with methyl green allowed for the simultaneous counting of autoradiographic positivity (five or more silver grains per nucleus) and PCNA positivity in the same nucleus. A PCNA antibody dilution curve demonstrated a wide range of dilutions (1:8000–1:1000), giving a stable ratio to the measured thymidine index. Lower dilutions (<1:250) produced much cytoplasmic and interstitial background staining. All single label PCNA antibody reactions were performed at a 1:4000 ratio; a 1:500 ratio was used for double immunocytochemistry.

Human Tissue Preparation. Major epicardial coronary artery segments were obtained from 13 diseased human hearts removed at the time of cardiac transplantation. Five of these hearts displayed severe coronary artery disease, and 8 hearts exhibited idiopathic dilated cardiomyopathy. Tissues were fixed overnight in methyl Carnoy's fixative, paraffin embedded, and sectioned. Separate arteries were snap frozen in OCT compound (Miles) and later sectioned for the Ki-67 antibody reactions described below. Artery segments were divided into two categories: (*i*) diffuse intimal thickening (DIT), in which the intimal thickness did not exceed the medial thickness and in which necrosis was absent; and (*ii*) atherosclerotic plaques, in which the intimal thickness exceeded the medial thickness and at least one focus of necrosis (often calcified) was present. Totally occluded segments or sections displaying thrombus or hemorrhage were excluded from the study. As control arterial tissue, portions of internal mammary artery not used for coronary bypass surgery were obtained and processed in the same way as the coronary artery segments. These human tissue studies received appropriate University of Washington Human Subjects Review approval.

Immunocytochemistry. Serial sections from each artery sample reacted with the following antibodies: anti-PCNA IgM (American Biotechnology, Plantation, FL; ref. 13); for double-label immunocytochemistry for PCNA and a cell type-specific antibody, HAM56 (anti-macrophage antibody at 1:4000 dilution; ref. 14); CD45 for lymphocytes and monocytes (1:20 dilution; Dakopatts); Mac 387 for neutrophils and macrophages (1:250 dilution; Dakopatts; ref. 15); HHF35 (to identify smooth muscle cells; ref. 16). Ki-67 antibody (Dakopatts; ref. 17) was used at a 1:50 dilution.

Single-label immunocytochemistry was performed by the immunoperoxidase technique (18) using strepavidin amplification (Jackson ImmunoResearch) with a light hematoxylin counterstain to visualize all nuclei in the tissue sections. Double-label immunocytochemistry was performed with the anti-PCNA antibody being developed by avidin-biotin-immunoperoxidase (ref. 18; Vector Laboratories), and with an alkaline phosphatase development of the cell type-specific antibodies (Vector Laboratories).

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Abbreviations: PCNA, proliferating cell nuclear antigen; DIT, diffuse intimal thickening.

The percentage PCNA-positive nuclei was obtained from adjacent ×100 microscopic fields of the arterial segments. Average percentages for each group of samples were obtained and compared by Student's *t* test.

RESULTS

Verification of the Proliferation Specificity of the PCNA Antibody. To confirm that in methyl Carnoy's fixed, paraffin-embedded tissues, anti-PCNA staining was proliferation specific, we studied normal tissues, in which the sites of proliferation are known. Sections of skin revealed a reaction product limited to hair follicle epithelial cells and to a few scattered basal cells of the epidermis. In the small intestine, positive labeling was limited to crypt epithelial cells as well as to a few scattered leukocytes of the mucosal interstitium. These distributions of proliferative activity were confirmed by separate [^{3}H]thymidine autoradiographs, using the single-pulse thymidine-labeling protocol. Tissue regions without thymidine labeling (i.e., upper epidermis and dermis, upper halves of intestinal villi) were also PCNA negative.

To study cell by cell correlations between anti-PCNA staining and thymidine labeling, simultaneous autoradiography and immunocytochemistry were performed on the same sections of rat tissues from animals sacrificed 1 hr after the single injection of [^{3}H]thymidine. In the small intestine, essentially all thymidine-positive cells were also PCNA positive (276/277 counted nuclei; Fig. 1*a*). However, PCNA labeled a greater fraction of the cells than did the thymidine labeling. Approximately 5 times as many cells were PCNA positive as were thymidine positive (1483 cells; overall PCNA index in tissue excluding muscularis, 25.1%). This is consistent with the restriction of thymidine labeling to cells actually in S phase, whereas PCNA protein is present in proliferating cells not only during S phase, but also during the G_1 and G_2 phases of the cell cycle (9, 19).

Arterial smooth muscle cells from the animals described above were also studied by comparing untraumatized carotid artery with vessels that had been balloon-injured prior to sacrifice. Again, PCNA labeling and thymidine labeling were correlated. The normal carotid artery contained no PCNA labeling (Fig. 1*b*) and essentially no thymidine labeling (0.15%), whereas the PCNA and thymidine labeling indices in the carotid artery 3 days after balloon injury were 17.3% and 9.8%, respectively (Fig. 1*c*). At 30 hr after balloon injury, all thymidine-positive cells were also PCNA positive (22/22 cells) but several more cells were PCNA positive and thymidine negative (159 cells; overall PCNA index, 19.1%).

Proliferation in Human Arteries. Six internal mammary arteries used as examples of nonpathologic human artery were without significant intimal thickening, in agreement with previous reports (20). In addition, almost no PCNA-positive cells were found (Table 1).

In the human coronary artery specimens (14 plaque specimens and 10 portions with diffuse intimal thickening), very few PCNA-positive cells were seen (generally 0–1% of cells;

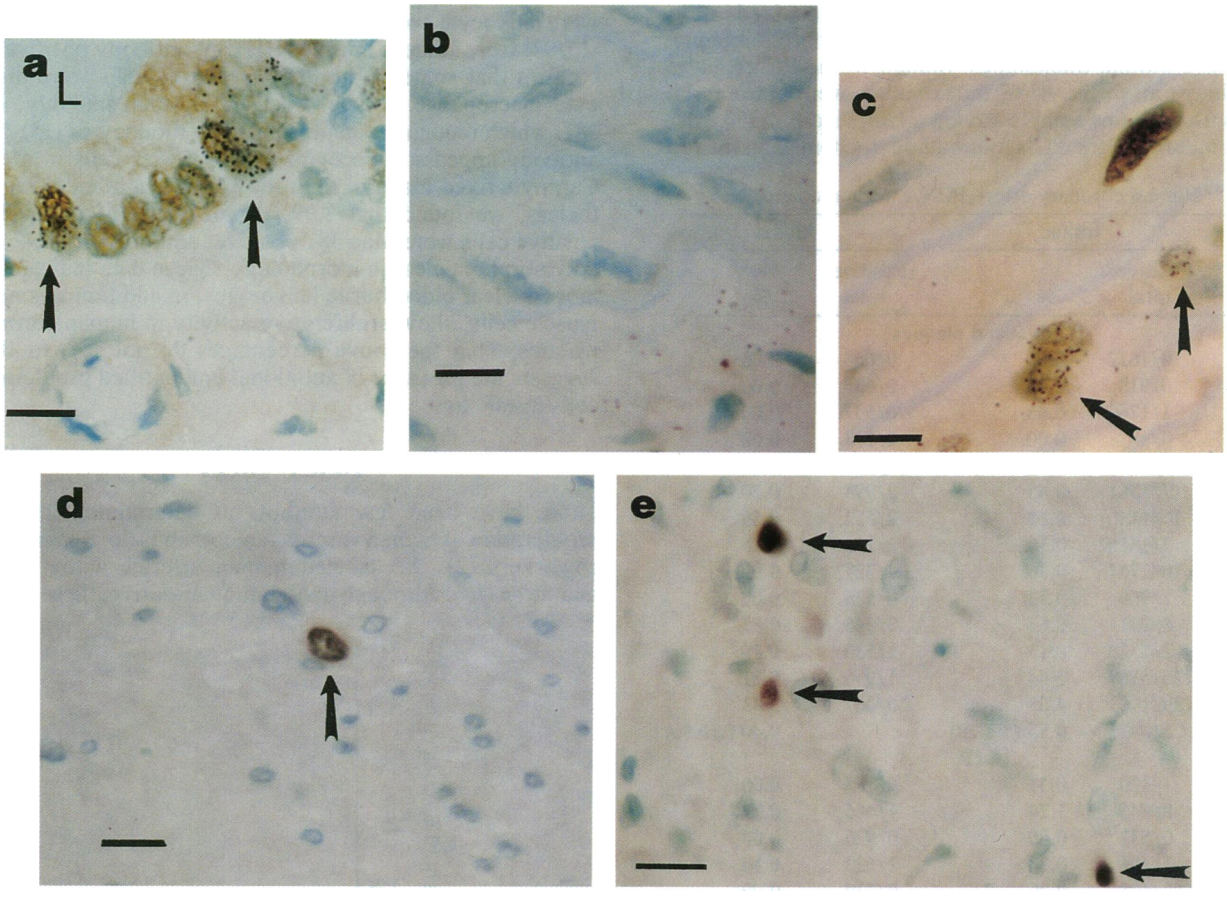

FIG. 1. (*a*) Combined [^{3}H]thymidine autoradiography and PCNA immunocytochemistry on rat small intestine. The nuclear labeling (brown reaction product) is limited to the crypt epithelial cells, two of which show superimposed silver grain clustering (arrows; L, crypt lumen). (*b* and *c*) Combined [^{3}H]thymidine autoradiography and PCNA immunocytochemistry on rat carotid arteries 30 hr after balloon injury. (*b*) The control, right carotid shows no PCNA and no thymidine labeling. (*c*) The injured carotid reveals scattered PCNA-positive nuclei, two of which are also thymidine labeled (arrows). (*d* and *e*) PCNA labeling of a plaque showing an isolated, positive intimal cell (*d*, arrow), and three positive intimal cells in a different intimal region (*e*, arrows). (Methyl green nuclear stain; bars = 10 μm.)

Table 1. Internal mammary artery PCNA nuclear counts

Patient	Intima Positive/total	Intima %	Media Positive/total	Media %
1	0/206	0	1/1259	0.1
2	0/51	0	2/589	0.3
3	0/227	0	2/798	0.3
4	0/292	0	0/1637	0.0
5	0/320	0	0/457	0.0
6	0/193	0	0/1313	0.0
	Mean (±SD)	0		0.11 (±0.15)

see Table 2 and Fig. 2 for summaries). Positive cells were often randomly located as single cells (Fig. 1*d*) with occasional clusters (Fig. 1*e*). No obvious pattern of localization with respect to plaque features was seen. Intima tended to have a slightly greater proportion of PCNA-positive cells than did media (mean ± SD, 0.62 ± 1.03 and 0.31 ± 0.71 for all intimas and medias, respectively), and plaque intima tended to have a higher PCNA index than did the diffuse intimal thickenings (mean ± SD, 0.85 ± 1.29 and 0.31 ± 0.40, respectively). However, none of these differences is statistically significant.

As an independent check on the range of labeling indices seen with the anti-PCNA antibody, some frozen sections of coronary arteries were examined with Ki-67. This antibody reacts with a different cell cycle-associated protein and is reported to react with proliferating cells throughout the cell cycle (11). The Ki-67 labeling indices are summarized in Fig. 3 for a separate series of coronary atherosclerotic plaques and diffuse intimal thickenings. Similar to the PCNA index data, almost all of the Ki-67 index values were in the 0–1% range with only one sample having a Ki-67 index >1%. Also in agreement with the PCNA immunocytochemistry, the few

Table 2. Human coronary artery PCNA nuclear counts

Patient	Intima Positive/total	Intima %	Media Positive/total	Media %
		Advanced plaques		
1	0/1612	0.00	0/668	0.00
2	0/918	0.00	0/635	0.00
3	1/1769	0.06	0/537	0.00
4	0/987	0.00	0/213	0.00
5	18/1333	1.35	1/492	0.20
6	7/1561	0.45	2/998	0.20
7	10/1136	0.88	2/552	0.36
8	3/1855	0.16	2/1361	0.15
9	10/2797	0.36	3/3273	0.09
10	7/960	0.73	0/536	0.00
11	6/931	0.64	3/346	0.87
12	2/1538	0.13	1/530	0.19
13	126/5169	2.44	1/956	0.11
14	89/1903	4.68	16/467	3.43
	Mean (±SD)	0.85 (±1.29)		0.40 (±0.90)
		DIT		
15	1/320	0.31	0/785	0.00
16	8/645	1.24	1/466	0.22
17	0/532	0.00	0/926	0.00
18	1/496	0.20	1/622	0.16
19	1/495	0.20	0/739	0.00
20	0/167	0.00	0/481	0.00
21	1/372	0.27	1/394	0.25
22	0/643	0.00	1/734	0.14
23	1/1062	0.09	2/1598	0.13
24	4/518	0.77	3/293	1.02
	Mean (±SD)	0.31 (±0.40)		0.19 (±0.31)

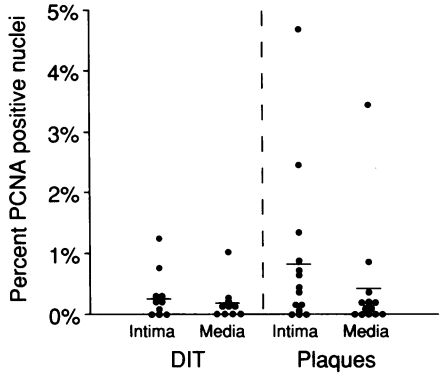

FIG. 2. Graph of PCNA indices per specimen, separated into intimal and medial indices for each sample of DIT and atherosclerotic plaques. Horizontal bars represent group averages.

positive cells appeared randomly dispersed either as single cells or as focal clusters of cells (data not shown).

Cell Types Displaying PCNA Reactivity. We also performed double-label immunocytochemistry with anti-PCNA and cell type-specific antibody on coronary plaques to identify the cell types displaying PCNA immunostaining. As expected from the low frequencies of PCNA labeling in this tissue, double-labeling cells were quite rare. Using four coronary specimens and pooling the counts for each cell-type antibody reaction, we found 27.1% (16/59) of the PCNA-positive cells were macrophages by HAM56 antibody staining, 21% (12/57) were leukocyte common antigen (CD45) positive, and 15.5% (15/97) were smooth muscle (HHF35) antibody positive. To confirm that some of the PCNA-positive cells were monocyte–macrophage in type, we used another antibody, Mac 387, which recognizes neutrophils and monocytes (15). This antibody appears to recognize many fewer cells in methyl Carnoy's fixed material than the HAM56 antibody. Nevertheless, we found that 3.9% (4/102 cells) of the PCNA-positive cells were Mac 387 positive, and none of these was polymorphonuclear in morphology. These data indicate that mononuclear blood-borne leukocytes, in addition to smooth muscle cells, show proliferative activity in human coronary plaques. That the above percentages do not sum to 100% suggests the presence of additional unidentified proliferating cells in this tissue.

DISCUSSION

There have been few attempts at determining levels of proliferation of human vascular smooth muscle. Spagnoli and coworkers (21, 22) labeled human arteries with [^{3}H]thymidine *ex vivo*. Although only semiquantitative, these studies

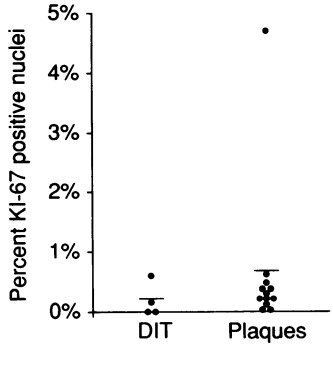

FIG. 3. Graph of Ki-67 antibody indices of intima, separated into DITs vs. atherosclerotic plaques. Horizontal bars represent group averages.

found a low level of replication in both the normal, adult artery wall (0–0.096%) and in plaque tissue. Questions of diffusion as well as other problems of thymidine incorporation into *ex vivo*-incubated tissue (11) make it difficult to be sure that this procedure reflects replication rates *in vivo*. Orekhov *et al.* (23) studied cells extracted from human aortic plaques with flow cytometry and found hardly any cells definitely in the DNA synthesis phase (S phase) of the cell cycle. Flow cytometry, however, measures DNA content rather than DNA synthesis. Moreover, cells obtainable by enzymatic dissociation may not be representative of the intact tissue.

Use of proliferation-specific antibodies is advantageous since neither *ex vivo* incubations of live tissue nor tissue dissociation is required, and proliferating cells are detected directly. Using protein gel electrophoresis and anti-PCNA antibodies, several studies show that the presence of PCNA protein is correlated with cell proliferation (9, 19, 24–26). Garcia *et al.* (10) suggested that the anti-PCNA antibody used in our study is effective on methyl Carnoy's fixed, paraffin-embedded material. However, no cell by cell proliferation correlation data were presented. There are reports of some PCNA mRNA being present in quiescent, cultured cells (27, 28). Also, Bravo and Macdonald-Bravo (29) had reported evidence for two forms of this protein, one of which was not clearly cell-cycle associated. For these reasons, it was necessary to first confirm the *in vivo* proliferation specificity of anti-PCNA staining by using our tissue preparation procedures and in experimental animals infused with [^{3}H]thymidine. After a single dose of [^{3}H]thymidine to label all cells in the S phase of the cell cycle, we found that essentially all thymidine-positive cells were also PCNA positive. The presence of many additional cells that were PCNA positive suggests that PCNA labeling occurs in the G_1 as well as the G_2 phases of the cell cycle, as has been reported in cell culture systems (9, 19, 25). Finally, given that the half-life of PCNA is reported to be ≈20 hr (29), it is probable that cells remain PCNA positive for some time after leaving S phase. PCNA should therefore be a more sensitive detection method for ongoing or recent cell replication than thymidine autoradiography, and it should be particularly useful when proliferation rates are expected to be very low, such as in the artery cell.

The parallel rates of replication shown by two cell-cycle-related proteins give greater confidence in the estimate of cell replication rates. Indices of PCNA and Ki-67-stained cells were low (usually <1%) in the artery walls and atherosclerotic plaques of human arteries. A similar low level was seen in uninjured, adult rat arterial media (0.04%; ref. 12). Replication in the plaque was orders of magnitude less than the 7–40% or higher rates reported for malignant human neoplasms (10, 11). Nevertheless, such low rates are consistent with the clinical observation that atherosclerosis usually takes several decades to produce clinically significant stenoses. Similarly, angiographic and ultrasound studies have shown that established plaques often remain unchanged over several years of observation (30, 31).

A few plaque sections revealed higher PCNA (or Ki-67) indices. Since only one section per plaque was analyzed in this study, it is not clear to what extent such variation represents sampling differences within single individual plaques versus true differences between individual intimal lesions. Nevertheless, these data raise several possibilities for individual human plaque growth, including (*i*) plaque growth is episodic, with periods of low level, indolent growth punctuated by brief episodes of greater proliferative rates; and (*ii*) individual plaques differ in their rates of growth, as has been suggested by some serial angiographic and Doppler ultrasound studies (30–32). At the moment, clinical imaging studies do not discriminate among the possibilities of episodic cell proliferation, thrombus formation, or hemorrhage into the plaque substance.

We were surprised to find significant PCNA staining of intimal cells identified by leukocyte-specific antibodies. The high frequency of HAM56 antibody-positive cells, plus the presence of Mac 387-positive cells, suggest that the majority of these leukocytes are monocyte–macrophage in type. However, in the absence of additional leukocyte subtype-specific antibodies, the possibility remains that some of the proliferating leukocytes are plaque lymphocytes (33, 34). In the carotid plaque study of Barrett and Benditt (35), fms mRNA was detected, associated with mRNA for platelet-derived growth factor B chain. Since fms mRNA encodes the colony-stimulating factor 1 (CSF-1) receptor of macrophages (36), plaque macrophages might themselves be proliferating under the influence of CSF-1. Other animal studies have shown that monocytes and macrophages outside of the bone marrow can divide (37, 38), and some studies have suggested a proliferative capability of arterial intimal macrophages. For example, based on hematoxylin and eosin staining and without benefit of immunocytochemistry, Villaschi and Spagnoli (22) reported that the rare thymidine labeling in plaques was "almost exclusively in focal infiltrates of foam cells and monocyte-like cells." Thymidine labeling and mitotic figures in intimal foam cells felt to be macrophages by light microscopy, ultrastructural, or immunocytochemical criteria have been reported by others in primate and rabbit models of atherosclerosis (39–41). Thus, two pathways leading to an increase in intimal macrophage mass appear to exist: (*i*) migration of monocytes from the blood stream into the intima (4, 42), and (*ii*) proliferation of macrophages within the plaque.

Although some smooth muscle cells and some macrophages in the tissues studied were PCNA positive, a large proportion of PCNA-positive cells were negative for the individual cell type-specific antibodies used. The nature of this suggested PCNA-positive but otherwise nonreactive cell population is not clear. Given that several studies have shown that smooth muscle cells can modulate to a phenotype that is deficient in smooth muscle-specific actin isotypes (43), it is possible that many such cells are actually smooth muscle in origin. Alternatively, the presence of an unidentified cell type may represent cells in the intima with special importance for the growth of this lesion. Recent studies with rat smooth muscle suggest that the arterial wall may contain cells derived from two distinct lineages (8). Finally, the combined use of proliferation-specific antibodies with markers for growth factors and their receptors should help to determine the significance of individual growth factor gene expression in the human artery wall (35, 44).

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