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Human Atherosclerosis

IV. Immunocytochemical Analysis of Cell Activation and Proliferation in Lesions of Young Adults

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The accumulation of smooth muscle cells is a major phenomenon associated with the pathogenesis of lesions of atherosclerosis. Smooth muscle cell proliferation in response to the release of growth factors from neighboring cells, both smooth muscle and macrophages, is one mechanism postulated to account for the increasing numbers of smooth muscle cells as atherosclerotic lesions progress. Indeed, we recently demonstrated the B chain of platelet-derived growth factor (PDGF-B), a potent smooth muscle mitogen, within macrophages in monkey and human lesions of atherosclerosis. To further test the hypothesis that smooth muscle proliferation and/or activation (eg, expression of major histocompatibility complex proteins) plays a role in the early development of these lesions, we applied antibodies to PDGF-B, HLA-DR (a marker of cell activation), and proliferating cell nuclear antigen (PCNA) (a cell proliferation-associated marker) on a series of early human atherosclerotic lesions from young adults in conjunction with cell-type-specific antibodies. Smooth muscle cells had previously been demonstrated to comprise a major fraction of the cell population in these lesions. In a continuing study of early and intermediate lesions of individuals ranging in age from 15 to 34 years, PDGF-B was detected within macrophages in 2 of 15 lesions. There was no evidence of HLA-DR expression by the smooth muscle cell population in any of the lesions. PCNA-positive cells comprised less than 2% of the cells in the lesions, and the majority of these were blood-borne cells (macrophages and/or lymphocytes), although a small fraction of the PCNA-positive cells were identified as smooth muscle. Concurrent PCNA and 5′-bromodeoxyuridine studies of peripheral blood monocytes demonstrated the presence of significant numbers of cells positive for these proliferation-related markers. It is concluded that the growth factor PDGF-B may have a role in regulating cell proliferation in early human fatty streaks, but the number of proliferating smooth muscle cells is relatively small, and there is no evidence of smooth muscle cell activation, as judged by HLA-DR positivity, in these lesions. (Am J Pathol 1993, 142:1787–1793)

In a recent study[1] we analyzed the cellular composition of some of the earliest grossly identifiable lesions of the human aorta, ie, the fatty streak. While previous studies had suggested a predominance of macrophages in these lesions,[2–6] our study, as well as one by Wissler et al,[7] provided evidence that these early-stage lesions contain a significant component of smooth muscle cells and smooth muscle cell-derived foam cells. Given the potential role of macrophage-derived growth factors such as platelet-derived growth factor (PDGF) in the proliferation of smooth muscle cells in atherosclerotic lesions,[8–10] it would be important to document the actual expression of such growth factors and the presence of concomitant cell proliferation and/or cell activation within human lesions. Such studies are now possible given the recent availability of monoclonal antibodies which can identify, in fixed, embedded tissues, the B chain of PDGF.[9] We therefore undertook a study to search for expression of the latter in the group of previously

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studied fatty streaks,[1] looking also at a marker of cell activation (HLA-DR), which has been associated with smooth muscle cell activation *in vivo* and *in vitro*,[11–14] and a marker of cell proliferation, proliferating cell nuclear antigen (PCNA), a cell cycle traverse-associated protein.[15–17]

Materials and Methods

Procurement of Tissue

Early lesions of atherosclerosis had been identified as linear, slightly raised yellow streaks in the ascending aorta in a series of 16 individuals (age range, 15–34 years) from the King County Medical Examiner's Office, as described previously.[1]

Immunocytochemistry

Specimen handling and tissue processing was as previously described.[1] Monoclonal antibodies used in this study, together with their specificities and working dilutions, are listed in Table 1.

For single labeling procedures, we used the avidin biotin[19] or streptavidin biotin[15] immunoperoxidase method, with nickel chloride-enhanced 3,3′diaminobenzidine as chromogen, yielding a black reaction product. For some antibodies (PGF007, HAM56, HHF35), an analogous avidin biotin immunoalkaline phosphatase technique was employed, with Vector Red (Vector Laboratories, Burlingame, CA) as chromogen, yielding a red reaction product.

For double labeling procedures, sequential avidin biotin immunoalkaline phosphatase and silver-enhanced immunogold (IGSS) procedures were employed as previously described.[9] For labeling of HLA-DR in combination with cell-type-specific antibodies (eg, anti-muscle actin antibody HHF35 or anti-macrophage antibody HAM56), immunostaining of HLA-DR was performed first using the indirect IGSS procedure, followed by the immunoalkaline phosphatase procedure. For immunolabeling with antibodies to PCNA or the PDGF-B chain in combination with cell-type-specific antibodies, immunostaining with anti-PCNA and anti-PDGF-B chain were performed first, using a streptavidin biotin immunoperoxidase procedure, followed by the immunoalkaline phosphatase procedure with the cell-type-specific monoclonal antibodies. When we performed double labeling with two antibodies of the same immunoglobulin class (eg, HLA-DR with HHF35, PDGF-B with HHF35), slides were treated with 0.10 mol/L glycine-HCl (pH 2.2) for 2 hours following the first immunostaining to elute free antibody to avoid cross-immunolabeling. Slides were washed overnight in phosphate-buffered saline at 4 C and then processed for immunolabeling with the second primary antibody.

For negative controls, primary antibody was replaced either with a normal mouse ascites fluid preparation, a normal mouse immunoglobulin fraction, or an irrelevant monoclonal antibody at matched protein concentration. All immunocytochemical preparations were counterstained with methyl green.

Peripheral Blood Studies

Thirty-ml specimens of peripheral blood were obtained by venipuncture from four young adult volunteers. The cells were incubated at 37 C for 1 hour in the presence of 100 μmol/L 5′-bromodeoxyuridine (BrdUrd). Following low-speed centrifugation of the whole-blood sample, the buffy coat containing the

Table 1. *Monoclonal Antibodies Used in Immunocytochemical Analyses*

Antibody	Specificity	Cells identified in vessel wall	Source	Working dilution
HHF35	Muscle actins	Smooth muscle cells	Dako Corp.*	1:8000†
HAM56	—‡	Macrophages	Dako Corp.*	1:2000†
2B11,PD7/26	CD45, CD45RB	Lymphocytes, monocytes	Dako Corp.*	1:80†
L26	CD20	B lymphocytes	Dako Corp.*	1:250†
OPD4	CD45RO	T lymphocytes	Ref. 18	1:500†
Leu22	CD43	T lymphocytes, monocytes, macrophages	B-D§	1:200†
19A2	PCNA	—	Coulter Lab‖	1:8000¶
LN3	HLA-DR	—	Biogenex Lab#	1:20†
PGF007	PDGF-B chain	—	Mochida Ph**	1:800†

* Dako Corporation, Carpinteria, CA.
† Via avidin biotin immunoperoxidase method.
‡ Antigen incompletely characterized.
§ Bectin Dickenson Laboratories, San Jose, CA.
‖ Coulter Laboratories, Hialeah, FL.
¶ Via streptavidin biotin immunoperoxidase method.
# Biogenex Laboratories, San Ramon, CA.
** Mochida Pharmaceutical, Japan.

white blood cells was identified, aspirated, and added to Ficoll-paque (Pharmacia Laboratories, Piscataway, NJ) for density gradient centrifugation in order to separate the mononuclear cells (eg, monocytes and lymphocytes) from neutrophils, reticulocytes, and contaminating red blood cells. The mononuclear component was immediately fixed in methacarn as a cell pellet. Following overnight fixation, specimens were processed and embedded in paraffin. Immunocytochemistry was then performed on these cell pellets as described above for tissue specimens. The total number of BrdUrd-positive and PCNA-positive nuclei on one slide from each subject's cell pellet were counted and recorded.

Results

HLA-DR Studies

In the fatty streaks obtained from the ascending aorta examined in these studies, HLA-DR identified a subpopulation of cells located predominantly in the superficial and middle portions of the lesions (Figures 1A, 1B, 1C). In double labeling experiments employing the cell-type-specific antibodies HHF35 and HAM56, HLA-DR expression was restricted to a subset of the HAM56-positive cells (Figures 1B, 1C). The HHF35-positive and the HLA-DR-positive cell populations were mutually exclusive (Figure 1A). As noted in our previous study,[1]

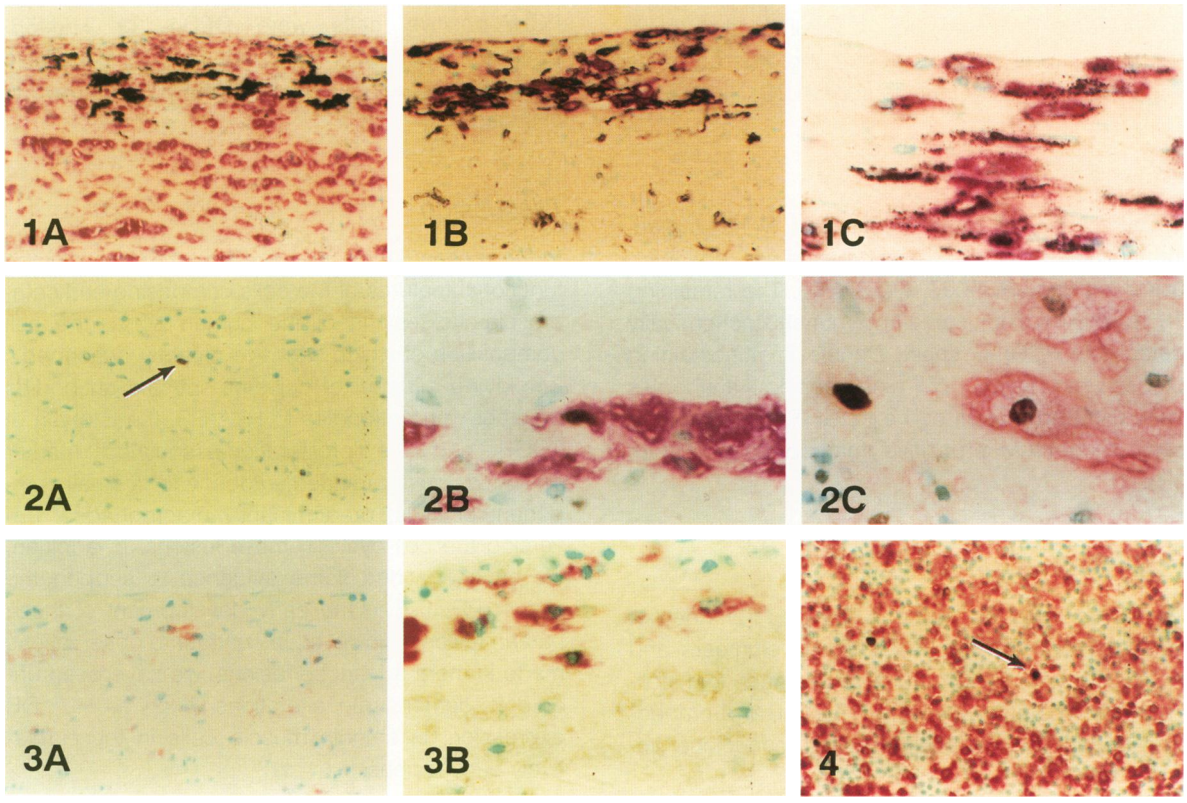

Figure 1. (Top) *Double label immunocytochemistry preparation showing distribution of cell-type-specific antigens (red) and HLA-DR (black) in fatty streak.* **A**, *nonoverlapping distribution of smooth muscle cells (HHF35 antibody, red) and HLA-DR (black).* **B,C**, *colocalization of anti-macrophage antibody HAM56 (red) and HLA-DR (black). Sequential avidin biotin immunoalkaline phosphatase and IGSS procedures with methyl green nuclear counterstain (see Materials and Methods). Original magnifications:* **A, B**, *×100;* **C**, *×400.*

Figure 2. *(Center) A, rare PCNA-positive cell nucleus noted in fatty streak (arrow); streptavidin biotin immunoperoxidase procedure.* **B,C**, *identification of PCNA-positive cells as macrophages (double label immunocytochemistry preparation using sequential streptavidin biotin immunoalkaline phosphatase and IGSS procedures; see Materials and Methods).* **B**, *Note PCNA-positive nucleus (black) corresponding to HAM56-positive cell (red).* **C**, *PCNA-positive nucleus (black) in HHF35-positive (red) smooth muscle-derived foam cell (right). PCNA-positive cell to the left is probably a macrophage. Original magnifications:* **A**, *×100;* **B,C**, *×400.*

Figure 3. *(Bottom) A, rare PDGF-B chain-positive cell noted in fatty streak (red). Streptavidin biotin immunoperoxidase procedure; original magnification, ×100.* **B**, *identification of PDGF-B chain-positive cells as macrophages (double label immunocytochemistry preparation using sequential avidin biotin immunoalkaline phosphatase and IGSS procedures; see Materials and Methods). Note PDGF-positive cells (black) corresponding to HAM56-positive cells (red). Original magnification, ×400.*

Figure 4. *(Bottom, right) Double label immunocytochemistry preparations on cell blocks prepared from peripheral blood buffy coats. Identification of some PCNA-positive cells (black nuclei) as HAM56-positive macrophages (red) [arrow]. Sequential streptavidin biotin immunoperoxidase and immunoalkaline phosphatase procedure (see Materials and Methods). Original magnification, ×100.*

the predominant cell type in these fatty streaks from the ascending aorta was smooth muscle cells (Figure 1A).

PCNA Studies

The number of PCNA-positive cells present in these lesions was small (Figure 2A). Less than 2% of the cells in all of the lesions examined were PCNA-positive. The PCNA-positive cells were observed principally in the most highly cellular lesions and almost exclusively within cells that appeared morphologically to be macrophages or lymphocytes. Occasionally, a PCNA-positive spindle-shaped cell could be observed. Double labeling immunocytochemistry studies demonstrated that the majority of PCNA-positive cells were macrophages (average, 78%) and/or blood-borne CD45-positive cells (average, 67%) (Table 2; Figure 2B). A small fraction (average, 14%) were HHF35-positive and thus could be identified as smooth muscle (Figure 2C).

PDGF-B Studies

In two of the 15 fatty streaks that were studied, intracellular PDGF-B chain protein was observed using antibody PGF007 (Figure 3A). The remaining 13 lesions did not contain immunohistochemically detectable PDGF-B chain. Double immunostaining procedures using cell-type-specific antibodies demonstrated that all of the PDGF-B chain-positive cells were HAM56-positive macrophages (Figure 3B).

Peripheral Blood Studies

Monocytic white cell fractions obtained from the peripheral blood of four young adult volunteers and incubated with BrdUrd for 1 hour prior to Ficoll-paque® separation, pelleting, fixation, and embedding in paraffin were evaluated with monoclonal antibodies to PCNA and BrdUrd; results are summarized in Table 3. Significant numbers of both PCNA-positive and BrdUrd-positive cells were noted, although far more PCNA-positive than BrdUrd-positive cells were observed. The average ratio of these two cell populations, respectively, was 8.47:1, with a range of 7.88 to 9.47. To identify the nature of the PCNA- and BrdUrd-positive cells, double labeling studies were performed, with results summarized in Table 4 and illustrated in Figure 4. Approximately 30% of the BrdUrd-positive cells were positive with either antibody OPD4 or HAM56, suggesting that they represented either T lymphocytes or macrophages. The exact nature of the remaining BrdUrd-positive cells was not determined. While all were CD45-positive, none were identified by the antibodies L26 (anti-CD20) or Leu22 (anti-CD43) (data not shown). Similar fractions of the PCNA-positive cells were OPD4- or HAM56-positive. Thus, the majority of the PCNA-positive cells were not identified with the antibodies used in this study.

Table 2. *Immunoreactivity of Intimal Cells in Fatty Streaks*

Case number (no. of lesions analyzed)	HAM56+/ PCNA+	HHF35+/ PCNA+	CD45+/ PCNA+
3954 (4)	16/22	2/18	13/20
6561 (3)	7/11	3/9	5/9
6624 (2)	8/9	1/7	6/9
6735 (1)	4/5	0/4	2/3
7041 (3)	9/9	1/10	11/11
7901 (2)	28/36	5/35	25/41
Total	72/92 (78%)	12/83 (14%)	62/93 (67%)

Discussion

In the present study, we have analyzed some of the functional features of the cells comprising early atherosclerotic lesions of the human aorta, the cell composition of which we have recently described.[1] Employing a panel of cell-type-specific antibodies together with antibodies to HLA-DR, the B chain of PDGF, and the proliferation-associated marker PCNA, we have provided evidence for a small component of cells, predominantly macrophages, traversing the cell cycle in these lesions. In addition, we have provided some evidence to support the role of PDGF-B in the early development of these human lesions. However, in contrast to observations made in more advanced lesions, we have been unable to demonstrate alterations, such as HLA-DR expression, in smooth muscle cells in these relatively early lesions.

Table 3. *Comparison of Two Measurements of Cell Proliferation in Normal Peripheral Blood Leukocytes from Young Adults*

Sample	PCNA	BrdUrd	Ratio*
A	1640	173	9.47
B	448	56.8	7.88
C	534	65.4	8.17
D	1230	147	8.35
Mean			8.47

* Ratio of total number of PCNA-positive cells to BrdUrd-positive cells.

Table 4. *Identification of PCNA- and BrdUrd-positive Cells in Peripheral Blood*

	PCNA+		BrdUrd+	
Sample	HAM56+	OPD4+	HAM56+	OPD4+
A	7.74%	19.5%	18.8%	11.5%
B	7.55%	20.6%	13.8%	13.8%
C	7.15%	21.0%	19.5%	12.9%
D	5.93%	28.4%	17.8%	20.4%

No Significant Class II HLA-DR Expression Is Present in Smooth Muscle Cells of Early Human Lesions

Expression of HLA-DR, as a representative class II antigen of the major histocompatibility complex, has been demonstrated in activated lymphocytes as well as smooth muscle cells, the latter in response to stimulation with tumor necrosis factor and/or γ-interferon *in vitro*.[13,14] In contrast to the findings of Jonasson et al[11] and Hansson et al,[12] who studied more advanced lesions such as fibrous plaques, there was no significant expression of HLA-DR by smooth muscle cells in the fatty streaks of the ascending aorta in this study. There are several possible explanations for these divergent results. First, it is possible that smooth muscle "activation," as indicated by neoexpression of HLA-DR antigen, occurs later during lesion development. Although the lesions we have examined, in contrast to the advanced lesions studied by Jonasson et al[11] and Hansson et al,[12] were grossly identified as fatty streaks in young adults, they appeared microscopically to correspond to advanced fatty streaks or fibrofatty lesions.[1] In a more recent study,[20] Xu et al confirmed that the number of HLA-DR-positive smooth muscle cells increases with the progression of lesions and, in particular, in association with the presence of activated lymphocytes. This is consistent with the mechanism proposed by Hannson et al,[12] who postulate cytokine release by lesion-associated T lymphocytes. The relative paucity of CD45-positive lymphocytes in the lesions in this study may account for the absence of HLA-DR-expression in the smooth muscle component. However, even in regions within fatty streaks that were relatively rich in lymphocytes, class II HLA-DR expression by the population of smooth muscle cells was not observed. A second possibility is that the lesions examined, which were restricted to the ascending aorta, may not correspond to those that will progress to more advanced atherosclerotic plaques, such as those in the abdominal aorta, where more advanced atherosclerotic lesions are more prevalent. In this scenario, the absence of HLA-DR-positive smooth muscle cells would be indicative of self-limited lesions.

The Principal PCNA Positive Cell Component of These Early Human Lesions Is Blood-Borne Cells, eg, Macrophages and Lymphocytes

The data presented here demonstrate that the vast majority of the PCNA-positive cells in these fatty streaks and fibrofatty lesions are HAM56-positive monocyte/macrophages and/or CD45-positive lymphocytes. While some PCNA-positive cells were HHF35-positive smooth muscle cells, these are decidedly in the minority. In descending order of frequency within the lesions, the PCNA-positive cell types are macrophages, T lymphocytes, and smooth muscle cells. These data are supported by those recently reported by Gordon et al[21] in more advanced human carotid lesions, as well as our preliminary observations of PCNA and cell-specific markers in more advanced aortic and carotid lesions (Katsuda et al, manuscript in preparation).

PCNA, a cell cycle traverse-associated protein, has been used by many investigators as an immunocytochemical marker of cell proliferation. In the case of lymphocytic cells, however, the published data suggest that PCNA expression is not restricted to actively proliferating cells. For example, Kurki et al[22] mapped the expression of PCNA as a function of T-cell stimulation, finding PCNA to represent a discrete step in T-cell activation, while more recent studies by Giordano et al[23] have confirmed increased expression of PCNA in late G_1, S, as well as G_2/M normal and leukemic lymphocytic cells. In the current study, PCNA expression was present in a substantial fraction of peripheral lymphocytes and monocytes in the young adults sampled; these numbers were almost an order of magnitude greater than the fraction of S-phase cells as determined by BrdUrd uptake. These results are reminiscent of our recent findings in cell lines *in vitro*[24] and those of Gordon et al,[21] where similar discordances between PCNA expression and BrdUrd or tritiated thymidine incorporation were evident. Identification of PCNA-positive cells in the peripheral blood raises the possibility that PCNA-positive T lymphocytes and monocytes may have migrated into the atherosclerotic lesions, retaining their positivity for an unknown interval of time. Clearly, further work will be required to define the "half-life" of the PCNA expression in these migrating cell populations and to as-

certain the applicability of this marker for proliferating cells in pathologic processes other than neoplasia.

Regardless of the interpretation of its significance, we have documented PCNA expression in only a small number of smooth muscle cells, despite the fact that they comprise the majority cell population in these lesions.[1] This may reflect the fact that the proliferative fraction of these cells is very low and/or that smooth muscle cell proliferation may be episodic. In either case, the "snapshot" of proliferation activity afforded by this method may not accurately assess cell proliferation in these lesions. Other potentially confounding factors that may account for the relatively large number of smooth muscle cells in the intima include cell migration or the possibility of pre-existing intimal cell masses.[25–27]

The B Chain of PDGF Can Be Demonstrated in Macrophages in Only a Small Number of Human Fatty Streaks

Finally, in this paper we demonstrate that the B chain of PDGF can be found in these early human lesions. A smaller fraction of the lesions contained immunoreactive PDGF-B chain than was observed in our previous study of nonhuman primate lesions[9]; there may be several factors accounting for this difference. First, the lesions in the current study were obtained from ascending aorta, which is a region in which more advanced atherosclerotic lesions are less likely to develop than in the abdominal aorta. Our failure to demonstrate significant numbers of PDGF-B chain-positive cells in these lesions may, in fact, be indicative of the self-limited nature of these lesions. Additional studies will have to be performed on comparable lesions in the abdominal aorta to test this hypothesis. A second explanation is simply that PDGF B chain plays a role later in the development of atherosclerotic lesions, ie, those more advanced than the lesions studied here. In this case, one would predict that the fraction of cells that can be identified with antibodies to PDGF-B chain would increase with lesion development. Another possible cause for the differences observed is the relative lability of this antigen and its subsequent loss in the human tissues studied owing to unavoidable postmortem intervals ranging from 2 to 22 hours in this Medical Examiners Office-derived case material. In contrast, the monkey lesions[9] were optimally fixed immediately upon the death of the animal. We are currently extending these studies in an attempt to obtain tissues from different areas in the aorta with an even shorter postmortem interval. In addition, it is possible that *in situ* hybridization for B chain mRNA may yield additional information.[28]

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