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Proliferating Cell Nuclear Antigen Immunohistochemistry in Rat Aorta After Balloon Denudation

Comparison with Thymidine and Bromodeoxyuridine Labeling

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The authors compared detection of proliferating vascular smooth muscle cells (SMC)* in vitro *and* in vivo *with proliferating cell nuclear antigen (PCNA) immunohistochemistry and two established methods: [^{3}H]thymidine autoradiography and bromodeoxyuridine immunohistochemistry. Labeling with [^{3}H]thymidine and bromodeoxyuridine of rat vascular SMC in culture stained 11% ± 2% and 11% ± 1% of cells, and PCNA immunohistochemistry 22% ± 2% of cells. Proliferation in the media of the denuded rat aortae was highest 3 days after denudation: 4.2%, 3.8%, and 4.7% of labeled cells with [^{3}H]thymidine, bromodeoxyuridine, and PCNA, respectively. In the intima, proliferation was highest 7 days after denudation with 42% [^{3}H]thymidine, 40% bromodeoxyuridine, and 46% PCNA-positive cells. With double labeling, all [^{3}H]thymidine-positive cells were PCNA positive, whereas some cells were only positive for PCNA. The authors conclude that PCNA immunohistochemistry compares favorably with [^{3}H]thymidine autoradiography, and bromodeoxyuridine immunohistochemistry. (Am J Pathol 1992, 141:685–690)

Proliferation of medial smooth muscle cells (SMC) is largely responsible for neointimal thickening during repair of arterial injury. [^{3}H]thymidine labeling, introduced in the 1950s, is the "gold standard" for cell kinetic studies.[1] Synthesis of DNA also can be detected immunochemically by identifying the incorporation of a thymidine analog bromodeoxyuridine using specific antibodies.[2] Because both methods require incorporation of the labeling molecule into the DNA during S phase, prior incubation of cells and tissues or *in vivo* administration is necessary.

The recent development of monoclonal antibodies to proliferation-associated antigens offers promise for the detection of proliferating cells in tissues previously harvested from experimental animals or humans.[1] Either fresh frozen or appropriately fixed tissues may be used.[3]

The sensitivity and specifity of these new immunohistochemical methods for detection of proliferating cells in vascular tissues after injury is not established. In this study we compared [^{3}H]thymidine labeling and bromodeoxyuridine immunohistochemistry of proliferating vascular SMC in cultures and in rat aorta after balloon denudation, with immunohistochemical detection of proliferating cell nuclear antigen (PCNA).

We report that PCNA immunostaining of cultured vascular SMC and of fixed, paraffin-embedded rat aortic tissues before and after balloon denudation, is more sensitive for detection of proliferation compared with the classical autoradiographic labeling index with [^{3}H]thymidine or to immunohistochemistry for bromodeoxyuridine.

Methods

Cell Cultures

Smooth muscle cells were isolated from the media of noninjured thoracic aortae of male Sprague-Dawley rats (200 to 250 g). After digestion with 0.05% elastase and 0.1% collagenase, the filtrate was centrifuged and the pellet resuspended in Waymouth's MB 752/1 medium

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supplemented with 5% fetal calf serum, l-glutamine, and antibiotics and grown until confluency. All cell lines were passages 5 through 10. Cells were seeded in Lab-Tek Flaskette Chambers slides (Nunc Inc., Naperville, IL) and incubated at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. After attachment, the media were replaced with serum-free medium for 24 hours. Cells then were incubated for 2 hours with either 25 μCi/5 ml [^{3}H]thymidine or with 150 μg bromodeoxyuridine (Amersham, Arlington Heights, IL). Slides incubated with [^{3}H]thymidine were washed in phosphate-buffered saline (PBS), dehydrated, and processed for autoradiography as described below. For bromodeoxyuridine immunostaining, cells were washed in PBS and fixed in acid ethanol. For PCNA immunostaining, cells were washed in PBS, placed in 1% paraformaldehyde in PBS for 2 minutes, and in methanol at −20°C for 10 minutes and washed in PBS before antibody incubation.

Animals

Adult, male Sprague-Dawley rats (400 to 500 g) were anesthetized with sodium pentobarbital (20 mg/kg intraperitoneally) and additional volatile anesthesia with isofluorane if necessary. The left iliac artery was exposed and an F2 embolectomy catheter was advanced to the aortic arch. The inflated catheter was withdrawn into the abdominal aorta three times. This procedure results in complete deendothelialization of the thoracic aorta with minimal necrosis of medial SMCs, as described previously.[4]

Rats were injected with 0.5 mCi/kg [^{3}H]thymidine or 30 mg/kg bromodeoxyuridine at 18, 6, and 1 hour before being killed at 1, 3, 7, and 14 days after balloon denudation.

The removed aortae were immersed in 3% glutaraldehyde and serially sectioned for light microscopy and immunohistochemical studies. Aortic segments were further fixed in 10% neutral buffered formalin for 12 to 24 hours, dehydrated in graded ethanol, and embedded in paraffin at 59°C. Sections were cut 4 μm thick and mounted.

Neointimal Proliferation

For the assessment of neointimal proliferation, 4-μm–thick full cross-sections (n = 4 rats in each group per time point, three sections per each aorta) were photographed at 10× magnification, and the intimal and medial area measured with a planimeter (Numonics, Montgomeryville, PA). The extent of neointimal proliferation was expressed as the ratio of intimal to medial area (mean ± standard error of the mean for each time point).

Thymidine Autoradiography

Slides were dipped in Kodak NTB-2 emulsion and developed after 2 weeks at 4°C. Sections then were lightly counterstained with hematoxylin and eosin.

Bromodeoxyuridine Immunohistochemistry

Slides were pretreated with 10% sheep serum, washed three times in PBS, and incubated for 90 minutes with a mouse anti-bromodeoxyuridine monoclonal antibody (Amersham, Arlington Heights, IL). The slides were washed in PBS three times and incubated for 30 minutes with peroxidase conjugated mouse antibody, and after washes in PBS, stained with diaminobenzidine tetrahydrochloride (Amersham, Arlington Heights, IL) and counterstained with diluted eosin and rehydrated.

PCNA Immunohistochemistry

Slides were pretrated with 10% normal horse serum, washed three times in PBS, and incubated with mouse monoclonal anti-PCNA antibody PC 10 (Dako, Santa Barbara, CA) at 1:20 dilution for 1 hour. After 3 PBS washes, sections were incubated for 30 minutes with biotinylated rabbit anti-mouse immunoglobulin. After repeated washes with PBS, slides were incubated with avidin and biotinylated horseradish peroxidase (1:200) for 30 minutes.

Diaminobenzidine tetrahydrochloride was used as chromogen. After repeated PBS washes, slides were counterstained in diluted hematoxylin and rehydrated. Sections of human lymph node with germinal centers served as positive control slides (Figure 1A).

All steps of immunohistochemistry were performed at room temperature in a humidity chamber.

Scoring of Autoradiography and Immunostaining

Three representative sections from aortae of 4 rats were scored for each time point. Slides were examined at 400× magnification; cells were counted as positive with 5 or more grains for thymidine autoradiography and when distinctly stained for immunohistochemistry. Intimal and medial cells were counted in one cross section, multiplied by 3 to give an estimate of total number of cells

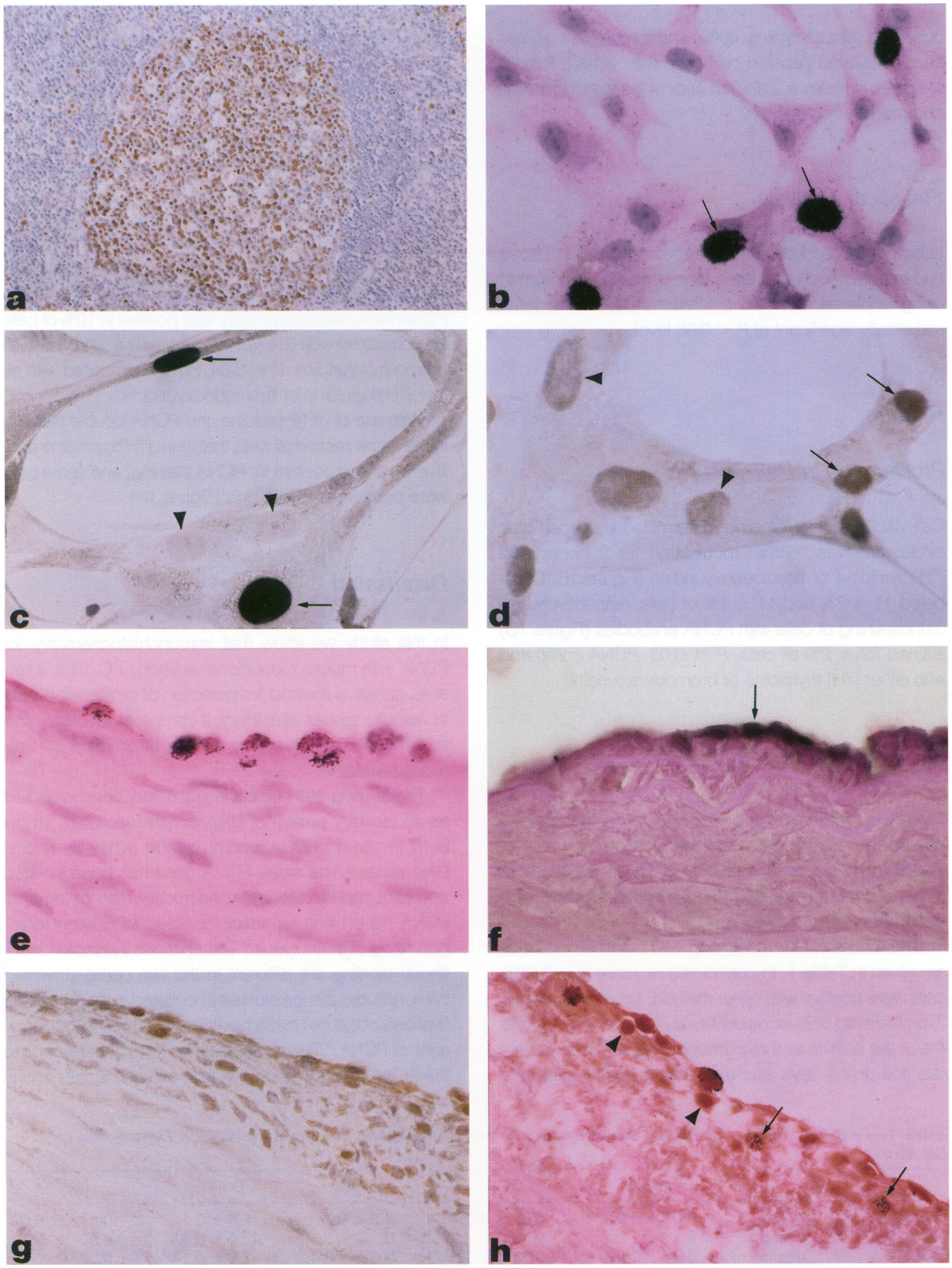

Figure 1. a: *PCNA-positive cells in the germinal center of a human lymph node (positive control) (original magnification ×40, hematoxylin counterstain).* b: *Thymidine-labeled nuclei in cultured vascular SMC (arrows) (original magnification ×600, hematoxylin, eosin counterstain.* c: *Bromodeoxyuridine-positive (arrows) and negative (arrowheads) nuclei in cultured vascular SMC (original magnification ×600, eosin counterstain).* d: *PCNA-positive (arrows) and negative (arrowheads) nuclei in cultured SMC (original magnification ×600, hematoxylin counterstain).* e: *Positive-thymidine-labeled cells in rat aorta 3 days after denudation (original magnification ×100, eosin counterstain).* f: *Positive bromodeoxyuridine-labeled cells in rat neointima 7 days after denudation (arrow, original magnification ×100, eosin counterstain).* g: *PCNA positivity in neointimal cells of rat aorta 14 days after denudation (original magnification ×100, hematoxylin counterstain).* h: *Double-labeled neointimal cells which are positive for both thymidine incorporation and PCNA (arrow) or for PCNA only (arrowheads) (original magnification ×100), eosin counterstain).*

observed (approximately 4000) and the percent labeled cells calculated (labeled cells/total cells ×100). Results are given in mean ± standard error of the mean for intima and media separately.

Statistical Analysis

Labeling indices from the three methods in cell cultures and in aortic section at each time point were compared using unpaired *t*-test. All differences were considered statistically significant at $P < 0.05$ level.

Results

Proliferation in Cell Cultures

Cell viability before labeling, determined by trypan blue exclusion, was >95%. Incubation for 2 hours with [^{3}H]thymidine or bromodeoxyuridine (Figure 1B, C) labeled 11 ± 2% and 11 ± 1% of cells, respectively. Immunostaining of cells with PCNA antibodies (Figure 1D) stained 22 ± 2% of cells, $P = 0.03$, PCNA compared with either [^{3}H] thymidine or bromodeoxyuridine.

Proliferation in Rat Aortae

There was no neointimal proliferation in control rats at 1 day and minimal 3 days after denudation. The ratio of intimal to medial area was 0.02 ± 0.002 7 days after denudation and increased to 0.27 ± 0.01 14 days after denudation.

The results of *in vivo* labeling with the three methods are given in Table 1. In control rats no more than 0.03% cells were positive with either method. On day 1 and day 3, proliferating cells increased to approximately 0.1% and 4% of the cells in all three groups (Figure 1E). In control rats and until 3 days after denudation, proliferating cells were detected only in the media. Seven days after denudation, the proliferation declined to 1% in the media, whereas more than 40% of the intimal cells were positive with all three methods (Figure 1F). There was a trend for higher percent positive cells with PCNA (46%), as compared with [^{3}H]thymidine (42)% and bromodeoxyuridine (40%), P = not significant.

Fourteen days after denudation, the proliferating activity decreased in the media and in the intima (Figure 1G). The percentage of proliferating cells in the media was 0.1% with all three methods, whereas in the intima PCNA immunohistochemistry was positive in 10% of cells as compared with 7% with [^{3}H]thymidine and 8% with bromodeoxyuridine, $P = 0.04$, PCNA compared with either [^{3}H]thymidine or bromodeoxyuridine.

With use of [^{3}H]thymidine and PCNA double labeling in the same section all cells that were [^{3}H]thymidine positive were also positive for PCNA staining, and some cells were positive for PCNA only (Figure 1H).

Discussion

In this study we show that immunohistochemistry for PCNA with mouse monoclonal antibody PC 10 is a reliable, sensitive method for detection of proliferating cells in vascular tissues after injury. It compares favorably with traditionally used labeling with [^{3}H]thymidine or bromodeoxyuridine.

Proliferating cell nuclear antigen has been identified as an auxiliary protein of DNA polymerase-delta,[5] most likely involved in DNA leading strand synthesis and in DNA repair.[6] The entire PCNA gene has been isolated and sequenced.[7] Several antigenically distinct forms of PCNA have been reported, localized to different compartments of the nucleus.[3] The epitopes available for antibody binding are different in the two compartments.[8] Immunofluorescence studies of cultured cells during the S phase of the cell cycle have indicated two subpopulations of PCNA.[9] The nucleoplasmic form corresponds to the PCNA present at low levels in quiescent cells and is

Table 1. *Percentage of Positive Medial and Intimal Cells for Each Labeling and Day After Balloon Denudation of Rat Aorta*

		Control	Day 1	Day 3	Day 7	Day 14
Thymidine	Media	0.01 ± 0.01	0.07 ± 0.01	4.2 ± 0.2	1.0 ± 0.1	0.17 ± 0.02
	Intima	0	0	0	42.0 ± 0.9	6.92 ± 0.71
BrdU	Media	0.01 ± 0.01	0.13 ± 0.03	3.8 ± 0.2	1.0 ± 0.1	0.12 ± 0.03
	Intima	0	0	0	40.1 ± 2.1	8.12 ± 0.51
PCNA	Media	0.01 ± 0.01	0.10 ± 0.01	4.7 ± 0.5	1.2 ± 0.1	0.16 ± 0.03
	Intima	0	0	0	46.0 ± 4.4	10.2 ± 1.00*

* $P = 0.04$ PCNA versus Thymidine, BrdU.
BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.

not apparent in cells fixed in organic solvents. The second form is associated with sites of DNA replication and cannot be extracted with organic solvents. For this reason the methods of tissue preparation and fixation and the antibodies used are of importance.[10–12] We fixed cells in cultures with paraformaldehyde and methanol; this protocol was found to give excellent results.[13] For the *in vivo* studies, we used routine formalin-fixed, paraffin-embedded sections, which give a positive signal with the PC 10 antibody.[3,14] The importance of duration of fixation should also be emphasized because studies by others[11] and in our laboratory (unpublished observation) show that the staining decreases or is abolished if fixation is prolonged for more than 48 hours.

Using the PCNA method, we found that the percentage of proliferating cells in cultures of vascular SMC was comparable to previous reports of studies using similar experimental conditions.[15] In normal rat aorta, cell replication rates were low.[16] We also found that DNA synthesis in the media was maximal 3 days after denudation and returned to normal 14 days after denudation. In the intima the replication rate was maximal 7 days after denudation, 10 times the rate of the synthesis in the media.[17]

In both the cultured rat aortic SMC in cultures and in the *in vivo* studies of rat aorta, before and after balloon denudation, PCNA immunohistochemistry detected a higher percentage of positive cells than either thymidine autoradiography or bromodeoxyuridine immunohistochemistry.[11,18] Proliferating cell nuclear antigen labeling occurs also in the G_1 and G_2 phase of cell cycle, and the half-life of PCNA was reported to be 20 hours.[9] It is therefore conceivable that cells remain PCNA positive even after leaving the S phase. In addition, [^{3}H]thymidine and bromodeoxyuridine incorporation occurs only during S phase, and some of the cells that were exposed only at the very end of S phase may pass into G_2 phase before fixation.[12] We found that in healing rat aorta all [^{3}H]thymidine-positive cells were PCNA positive. This close spatial and numerical correlation between methods of labeling validates the use of PCNA immunohistochemistry in detection of proliferating vascular SMC after injury.

In conclusion, PCNA immunohistochemistry appears to be a more sensitive method for detection of proliferating cells in vascular tissues than thymidine or bromodeoxyuridine incorporation. There are several practical advantages of the PCNA method: radioactive materials can be avoided and no *in vitro* incubations or *in vivo* injections are needed. Further, because certain growth factors can induce increased PCNA mRNA stability and PCNA expression,[19] proliferating cell nuclear antigen immunohistochemistry may provide additional information on the role of autocrine or paracrine growth factors in arterial wall healing after injury.

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