Replication in restenotic atherectomy tissue

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Abstract

Previously, we demonstrated that replication in restenotic coronary atherectomy specimens was an infrequent and modest event. In general, this data was interpreted with caution, as immunocytochemistry for the proliferating cell nuclear antigen (PCNA) was used to subjectively assess proliferation and most of the tissue specimens were resected more than 3 months after the initial interventional procedure. The purpose of the present study was to use a more sensitive method of detecting replication, in situ hybridization for histone 3 (H3) mRNA, to determine the replication profile of human directional atherectomy specimens. Restenotic directional coronary atherectomy specimens from lesions that had undergone an interventional procedure within the preceding 3 months were studied. In addition, larger atherectomy specimens from peripheral arterial lesions were assessed to ensure that pockets of replication were not being overlooked in the smaller coronary specimens. We found evidence for replication in tissue resected from 2/17 coronary and 9/12 peripheral artery restenotic lesions. In contrast, 3/11 specimens resected from primary lesions of peripheral arteries also expressed H3 mRNA. We estimated that the maximum percentage of cells that were replicating in restenotic coronary, restenotic peripheral and primary peripheral artery tissue slides to be ≤0.5, ≤1.2 and <0.01%, respectively. Replication was found in tissue specimens resected both early and late after a previous interventional procedure. For specimens with >15 replicating cells per slide we found high levels of focal replication. Therefore, cell replication, as assessed by the expression of H3 mRNA, was infrequent in restenotic coronary artery specimens, whereas peripheral restenotic lesions had more frequent and higher levels of replication regardless of the interval from the previous interventional procedure. For all specimens the percentage of cells that were replicating was low, however focal areas with relatively high replication indices were presented. Although replication was more abundant in restenotic lesions it does not appear to be a dominant event in the pathophysiology of restenosis. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Restenosis; Proliferation; Smooth muscle cell; Atherosclerosis; Histone

1. Introduction

Understanding the complex nature of cell replication in the pathogenesis of human atherosclerosis, and more importantly restenosis (RS) after percutaneous interventional procedures is of fundamental interest in vascular medicine. While current concepts suggest that shrinkage of the artery wall due to negative remodeling, as well as the accumulation of extracellular matrix proteins are key processes in the pathogenesis of primary and restenotic lesions, the exact role of proliferation in these processes cannot be discounted [1,2]. It is likely that the highest levels of smooth muscle cell replication occur within the first few months of life when the neointima undergoes a rapid but limited expansion [3,4]. Vascular cell proliferation also occurs in primary (PR) atherosclerotic lesions but at low levels...
[5,6]. Perhaps the most convincing data regarding cell replication is the documentation of monoclonal populations of smooth muscle cells in the artery wall. The Benditts studied inactivation of the X-linked enzyme glucose-6 phosphate dehydrogenase (G6PD) as a means of assessing monoclonality and found that most atherosclerotic plaques contained a single isoform of G6PD [7]. More recently, Murry and colleagues studied the monoclonality of atherosclerotic plaques using X chromosome inactivation patterns [8]. Using the polymerase chain reaction they examined the monoclonality of plaques using the methylation pattern of the human androgen receptor gene, a highly polymorphic locus on the X chromosome for which 90% of women are heterozygous. These investigators noted that diseased as well as normal arteries contain monoclonal populations (or patches) of cells. Therefore, they speculated that the monoclonality of plaques might be due to expansion of a pre-existing monoclonal patch of cells, rather than mutation or selection of individual cells in the artery wall.

In a previous study we examined the replication profile of RS atherectomy specimens as these lesions are thought to be the result of exuberant smooth muscle cell proliferation [6]. Using immunocytochemistry for the proliferating cell nuclear antigen (PCNA) we demonstrated infrequent and modest levels of replication in human directional coronary atherectomy specimens resected from RS lesions. These results were considered controversial, particularly since they differed from those of other investigators [9–11]. Moreover, the majority of the RS specimens that we studied were resected more than 3 months after the original interventional procedure when proliferation may have already diminished or ceased. Finally, as we will later discuss, there are limitations with all immunocytochemical methods that are available for the study of cell proliferation in human tissues.

The current study is a reassessment of the replicative profile of RS vascular lesions using different tissues and techniques. First, we studied RS coronary atherectomy tissue resected from lesions subjected to an interventional procedure within the preceding 90 days. Second, we included directional atherectomy tissue from peripheral arteries as well as coronary arteries, as tissue specimens from peripheral lesions are larger in size and contain more cells. Third, the expression of H3 mRNA was used to assess proliferation. H3 mRNA increases 30–50-fold when cells enter S phase of the cell cycle, and is degraded 35-fold within 2 h of the end of S phase [12]. The results of the present study parallel our previous data and demonstrate low levels of replication in all tissues. A new finding, however, is the presence of higher levels of focal replication in RS compared to PR plaques.

2. Methods

2.1. Positive control (proliferating) tissue

Two different sources of replicative tissue were used for positive control experiments. Rat small intestine is highly replicative particularly at the base of intestinal microvilli, and has been used in several studies as positive control tissue for proliferation [5,6]. Similarly, tissue from stenotic arteriovenous fistulae of hemodialysis patients contains a moderate number of cells in the adventitia of the graft that express the PCNA antigen [13]. We were able to obtain two such specimens from patients undergoing surgical revision of their stenotic grafts. Fresh specimens of both of these positive control tissues were immersion fixed overnight in 10% neutral buffered formalin and processed into paraffin blocks.

2.2. Human directional atherectomy tissue

After obtaining patient consent, directional atherectomy specimens were collected from the clinical interventional laboratories of the University of Washington Medical Center and Sequoia Hospital. Emphasis was placed on the collection of specimens from ‘early’ RS lesions and larger tissue samples. Therefore, from > 750 directional atherectomy specimens logged into our specimen bank we retrospectively identified RS coronary atherectomy specimens from patients who had undergone a previous coronary interventional procedure but developed clinical RS within 90 days of the procedure. As well, directional peripheral atherectomy specimens from RS lesions were used, as this tissue is approximately five times larger than specimens retrieved from coronary lesions. Directional peripheral atherectomy specimens from PR lesions were also studied for comparative reasons. All fresh specimens were immersion fixed in 10% neutral buffered formalin and processed into paraffin blocks before being sectioned at 5-μm intervals and applied to Superfrost Plus slides (Fisher, Nepean, Ont.).

2.2.1. RS coronary atherectomy specimens

A total of 17 directional coronary atherectomy specimens were collected from 13 male and four female patients (median age: 50; range: 34–73 years) with symptomatic coronary artery disease. Of these tissue specimens, 15 were from native coronary arteries, and two were resected from aorto-coronary saphenous venous bypass grafts. The interval from the most recent interventional procedure varied from 1 to 84 days, with a median interval of 51 days. Of the directional coronary atherectomy specimens, three were from lesions that had undergone an initial interventional procedure within the preceding 1, 9 and 13 days.
2.2.2. Primary and RS peripheral atherectomy specimens

A total of 23 directional atherectomy specimens were resected from 11 PR and 12 RS peripheral arterial lesions of 13 patients (two females, 11 males) with clinically significant lower extremity claudication. Of these patients six had tissue resected from more than one lesion, and one had five tissue specimens serially collected from two RS lesions over the course of 140 days. The median age of these patients was 69 years (range: 54–80 years). Of the 23 lesions, 20 involved the superficial femoral or iliac arteries. The remaining specimens were resected from popliteal and tibial arteries. For the 12 RS specimens, the interval from the previous interventional procedure varied from 2 days to 29 months. Two restenotic specimens were resected from lesions that had been treated by balloon angioplasty 6 and 9 years previously, however, it is more likely that the histopathology of these lesions was more akin to that of the PR lesions. Of the RS peripheral lesions, three had directional peripheral atherectomies performed 2, 31 and 33 days after an initial procedure.

2.3. In situ hybridization

The H3 cDNA used in this study was originally isolated from mouse myeloma cells by Marzluff and colleagues, and cloned into a pGEM3 transcriptional vector by Wong and colleagues [14,15]. Mouse and human H3 cDNA show a high degree of homology; moreover, in unpublished studies we have demonstrated that radiolabeled mouse cDNA strongly hybridizes with human tonsil RNA on a Northern blot. After linearizing the murine H3 plasmid with Sal I, an anti-sense riboprobe was generated using T7 polymerase and [33P]UTP. Labeled riboprobe transcripts were separated from unincorporated precursors using a Nick column (Pharmacia, Uppsala Sweden), precipitated in ethanol, and resuspended in hybridization buffer. Aliquots containing 500 000 cpm/µl were applied to each slide. Sense riboprobe was generated from an unrelated cDNA and labeled in an identical manner. Slides for each tissue specimen were hybridized with the antisense and sense riboprobes overnight at 55°C before being washed in a 2 × buffer of sodium chloride/sodium citrate followed by a high stringency solution of 0.1 × sodium chloride/sodium citrate at 65°C for 2 h. After overnight drying, slides were dipped in Kodak NTB2 emulsion and stored in light protected desiccated boxes at 4°C. The slides were developed 7 days later and counterstained with hematoxylin. Slides were manually scanned using dark field microscopy at × 100 magnification to identify silver grains in tissue areas with hybridization. To define which cells over-expressed H3 mRNA, we used the in situ hybridization results with the positive control tissues to establish arbitrary criteria for a positive hybridization signal (Section 3).

2.4. Immunocytochemistry

Adjacent tissue sections were immunolabeled with the following antibodies to assist in the identification of proliferating cells: anti-smooth muscle cell z-actin (1:100 dilution; Boehringer Mannheim, Indianapolis, IN) to identify smooth muscle cells; anti-CD-68 (1:50 dilution; DAKO-CD68, KP1, DAKOPATTS, Denmark, pre-digested with protease type XXIV at room temperature for 10 min) to identify macrophages and monocytes; and anti-HPCA-1 (CD-34) antibody (1:40 dilution, Becton Dickinson, San Jose CA, pre-digested with 1 × trypsin for 20 min) to identify endothelial cells. Primary antibodies were detected with a biotinylated anti-mouse secondary IgG antibody, followed by an avidin-biotin-peroxidase conjugate, and the standard peroxidase enzyme substrate, 3,3’-diaminobenzidine (DAB). Nickel chloride was added to yield a black reaction product, and methyl green was used as a nuclear counterstain.

2.5. Statistical analysis

All results are expressed as means ± S.D. Statistical significance was evaluated using Student’s t-test for comparison between two means. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Histone H3 expression in control tissue

To ensure that hybridization for H3 mRNA was specific for proliferating cells we hybridized radiolabeled H3 riboprobe with rat intestinal tissue and tissue from stenotic human arteriovenous fistulae. The intestinal tissue over-expressed H3 mRNA in the lower one third of the intestinal microvilli, similar to the distribution pattern seen with anti-PCNA antibody immunolabeling [5,6] (Fig. 1A,B). Similarly, the expression pattern of H3 mRNA in human tissue from arteriovenous fistulae paralleled the results reported by Rekhter and colleagues who used an anti-PCNA antibody to study proliferation in this tissue [13] (Fig. 1C). Cells on the adventitial side of these grafts over-expressed H3 mRNA. Immunolabeling studies performed on adjacent tissue slides indicated that both smooth muscle and endothelial cells were replicating in these specimens.

The in situ hybridization signal for these positive control tissues was distinct from the background signal. Cells that over-expressed H3 mRNA were easily identified using darkfield microscopy at × 100 magnification by the abundance of silver grains that developed in the radiosensitive emulsion overlying the tissue sections.
(Fig. 1). Therefore, for the atherectomy tissue described below we used dark-field microscopy at ×10 magnification to screen each slide for cells that hybridized with the radiolabeled H3 riboprobe. When a cluster of silver grains was identified over a tissue fragment, a specific hybridization signal was confirmed at ×400 magnification by the presence of an associated hematoxylin-stained nucleus.

3.2. Replicative profile of RS coronary atherectomy specimens

Only two of 17 RS coronary atherectomy specimens in this study had cells that expressed H3 mRNA. The specimen with the highest proliferation profile had 19 cells expressing H3 mRNA and was obtained 1 day after an inadequate angioplasty procedure. Immunolabeling of adjacent slides with cell identity markers revealed that all cells over-expressing H3 mRNA were smooth muscle cells (Fig. 2A–E). The RS coronary artery specimen with the second highest proliferation profile had only three cells expressing H3 mRNA, and was obtained 60 days after a directional atherectomy had been performed at the same site. From previous studies with RS directional atherectomy specimens we determined that the average total number of cells per slide is 4841 ± 4458 cells [6]. This number was arrived at after manually counting the total number of cells per slide for 19 RS directional coronary atherectomy specimens. Although the range of total cells per slide was broad, this number is still useful for estimating the orders of magnitude for the number of proliferating cells on a slide. Therefore, for the RS directional coronary atherectomy specimen with the greatest replication profile (19 cells expressing H3 mRNA) we estimated that <0.5% of all cells on this slide were replicating.

3.3. Replicative profile of primary and RS peripheral atherectomy specimens (Table 1)

The frequency of specimens with proliferating cells was much higher for tissue derived from peripheral compared to coronary lesions. For example, three of 11 PR and nine of 12 RS peripheral atherectomy speci-

![Fig. 1. Validation of in situ hybridization with radiolabeled histone H3 riboprobe on positive control tissue. (A) Dark field photomicrograph of hybridization result on rat small intestine tissue. The silver grains are localized at the base of the intestinal crypts. The lumen of the gut is to the right (magnification: ×200). (B) High powered photomicrograph of A showing specific hybridization over the epithelial cells at the base of the intestinal crypts (hematoxylin counterstain, magnification: ×400). (C) In situ hybridization result using human arterio-venous fistula. The graft material is located in the upper left of the photomicrograph. The graft adventitia is in the bottom half of the photomicrograph and there is positive hybridization of the histone riboprobe with select cells (hematoxylin counterstain, magnification: ×400).]
Fig. 2. Proliferation in a smooth muscle cell rich restenotic peripheral atherectomy specimen from patient ‘RS3’ resected 10 months after a previous procedure. (A) Hematoxylin and eosin stain of atherectomy specimen with a large tissue fragment (magnification: ×40). (B) Dark field photo micrograph showing in situ hybridization result with histone H3 riboprobe (magnification: ×40). There are scattered cells that have overlying silver grains, indicative of positive hybridization. (C) Hybridization with sense riboprobe showing lack of specific hybridization for the same tissue fragment (magnification: ×40). (D) Immunolabeling of tissue fragment with antibody to smooth muscle cell α-actin. The majority of the cells in the large tissue fragment are smooth muscle cells (hematoxylin counterstain, magnification: ×100). (E) High-powered photomicrograph showing specific hybridization of histone H3 riboprobe with smooth muscle cells. Silver grains predominate in the vicinity of the nuclei of three cells (hematoxylin counterstain, magnification: ×400).

mens contained cells that hybridized with the H3 riboprobe. In three of the PR lesion specimens only one cell per slide was expressing mRNA. In contrast, the maximum number of cells on a slide that over-expressed H3 mRNA in RS specimens was 118, with a median value of 17. Manual counting of the total number of cells on directional peripheral atherectomy tissue slides proved to be even more laborious than coronary artery specimens as these specimens are larger and contain many tissue fragments. We did, however, count the total number of cells per slide for six specimens and found 10 182 ± 2474 cells. Therefore, the PR and RS periph-
eral atherectomy specimens with the maximum number of cells expressing H3 mRNA per slide had replication profiles of approximately < 0.01 and 1.2%, respectively. It is important to note that the degree of proliferation in these specimens did not correlate with the acuteness of the RS interval. For example, the two RS specimens with the highest number proliferative cells per slide (e.g. 118 and 61 cells) were collected 2 days and 9 years, respectively, after a previous interventional procedure.

3.4. Patterns of cell replication

In order to study the spatial distribution of replicating cells in these specimens, coronary and peripheral atherectomy specimens with > 15 cells on a slide that over-expressed H3 mRNA were analyzed in detail. A total of six specimens met this criterion. For two specimens the replicating cells were widely dispersed in different tissue fragments, with only one or two cells that expressed H3 mRNA per tissue fragment. Each of the four remaining specimens had between one and seven tissue fragments with < 10 cells per fragment that over-expressed H3 mRNA. A total of 12 tissue fragments from the latter four slides were analyzed (Table 2). To quantify the focal nature of proliferation in these 12 tissue fragments we counted the total number of cells per fragment, and then determined the percentage of cells per fragment that were proliferating.

The percentage of cells per tissue fragment that expressed H3 was remarkably elevated (e.g. 0.98–6.64%; mean: 3.87%). However, the distribution of these prolif-

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### Table 1

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Artery</th>
<th>Sex</th>
<th>Age</th>
<th>No. H3+ cells/slide</th>
<th>RS interval</th>
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<td>Primary</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR1</td>
<td>SFA</td>
<td>M</td>
<td>71</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>PR2</td>
<td>TIB</td>
<td>M</td>
<td>71</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>PR3</td>
<td>ILIAC</td>
<td>M</td>
<td>63</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
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<td>POP</td>
<td>M</td>
<td>67</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
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<td>ILIAC</td>
<td>M</td>
<td>79</td>
<td>0</td>
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</tr>
<tr>
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<td>SFA</td>
<td>M</td>
<td>60</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>PR7</td>
<td>SFA</td>
<td>M</td>
<td>71</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>PR8</td>
<td>TIB</td>
<td>M</td>
<td>71</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
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<td>SFA</td>
<td>M</td>
<td>66</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>PR10</td>
<td>SFA</td>
<td>M</td>
<td>73</td>
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</tr>
<tr>
<td>PR11</td>
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<td>M</td>
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<tr>
<td>Restenotic</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RS1</td>
<td>SFA</td>
<td>M</td>
<td>72</td>
<td>118</td>
<td>2 days</td>
</tr>
<tr>
<td>RS2</td>
<td>SFA</td>
<td>M</td>
<td>73</td>
<td>61</td>
<td>9 years</td>
</tr>
<tr>
<td>RS3</td>
<td>ILIAC</td>
<td>M</td>
<td>75</td>
<td>43</td>
<td>10 months</td>
</tr>
<tr>
<td>RS4</td>
<td>SFA</td>
<td>M</td>
<td>72</td>
<td>35</td>
<td>105 days</td>
</tr>
<tr>
<td>RS5</td>
<td>SFA</td>
<td>M</td>
<td>72</td>
<td>19</td>
<td>33 days</td>
</tr>
<tr>
<td>RS6</td>
<td>SFA</td>
<td>M</td>
<td>72</td>
<td>17</td>
<td>31 days</td>
</tr>
<tr>
<td>RS7</td>
<td>SFA</td>
<td>M</td>
<td>72</td>
<td>15</td>
<td>105 days</td>
</tr>
<tr>
<td>RS8</td>
<td>SFA</td>
<td>M</td>
<td>66</td>
<td>9</td>
<td>6 years</td>
</tr>
<tr>
<td>RS9</td>
<td>SFA</td>
<td>M</td>
<td>80</td>
<td>8</td>
<td>9 months</td>
</tr>
<tr>
<td>RS10</td>
<td>SFA</td>
<td>M</td>
<td>68</td>
<td>0</td>
<td>29 months</td>
</tr>
<tr>
<td>RS11</td>
<td>SFA</td>
<td>M</td>
<td>75</td>
<td>0</td>
<td>7 months</td>
</tr>
<tr>
<td>RS12</td>
<td>SFA</td>
<td>F</td>
<td>80</td>
<td>0</td>
<td>8 months</td>
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* No. H3+ cells, number of proliferating cells that hybridize with histone H3 riboprobe; POP, popliteal artery; SFA, superficial femoral artery; TIB, tibial artery.

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### Table 2

<table>
<thead>
<tr>
<th>Specimen</th>
<th>RS interval</th>
<th>Total no. H3+ cells/slide</th>
<th>No. tissue fragments with &gt;10 H3+ cells</th>
<th>Total no. tissue fragments/slide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA1</td>
<td>1 day</td>
<td>20</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>RS1</td>
<td>2 days</td>
<td>305</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>RS2</td>
<td>9 years</td>
<td>61</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>RS3</td>
<td>300 days</td>
<td>52</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>12</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

* DCA, directional coronary atherectomy specimen; No. H3+ cells, number of proliferating cells that hybridize with histone H3 riboprobe.
Fig. 3. Proliferation of macrophages in restenotic peripheral atherectomy specimen from patient ‘RS1’ resected 2 days after a previous procedure. (A) Hematoxylin and eosin stain of a tissue fragment (magnification: ×100). (B) Immunolabeling of tissue fragment found in A with an anti-CD68 antibody to demonstrate the abundance of monocytes/macrophages in this tissue fragment (magnification: ×100). (C) Dark field photo micrograph demonstrating abundant hybridization of histone H3 riboprobe with cells (magnification: ×100). (D) Sense control hybridization demonstrating the absence of specific hybridization signals (magnification: ×100). (E) High powered photo micrograph demonstrating specific hybridization of histone H3 riboprobe with cells in this macrophage rich tissue fragment (hematoxylin counterstain, magnification: ×400).

The proliferative tissue fragments in these four tissue slides was very patchy. Overall, we found that only 12/51 (or 24%) of all tissue fragments in these four specimens contained >10 cells expressing H3 mRNA.

Next, we studied the identity of the cells that were proliferating. Unfortunately, it was often difficult to identify the proliferating cells because a cell that expressed H3 mRNA on one slide was often absent on an adjacent tissue section. Therefore, the immunolabeling of cells on adjacent tissue slides revealed the nature of cells present in a specific region of a tissue block, but not necessarily the exact same cell that expressed H3 mRNA. Fig. 2A–E and Fig. 3A–E show examples of tissue specimens with foci of replication that involve...
smooth muscle cells and monocytes/macrophages, respectively. Of 12 tissue fragments, ten contained a mixture of replicating endothelial cells, smooth muscle cells, and monocytes/macrophages, while two tissue fragments were devoid of replicating endothelial cells. In the ten fragments that contained replicating endothelial cells, the endothelial cell replication index ranged from 0.16 to 2.95% (mean: 1.24%). The replication profile of all other (non-endothelial) cells varied from 0.98 to 5.10% (mean: 2.83%).

4. Discussion

This study examined cell proliferation in atherectomy tissue resected from human arterial lesions. The principal results are as follows: (1) proliferation is infrequent in tissue resected from RS coronary artery lesions; (2) replication is more frequent and higher in RS compared to PR peripheral artery lesions; (3) for all specimens the percentage of cells on a tissue slide that are proliferating is low; and (4) relatively high focal levels of proliferation were present in a minority of specimens.

The study of replication in human arteries is crucial for advancing our understanding of not only the processes involved in atherogenesis but also various forms of accelerated lesion formation such as RS. A major hurdle for these studies is the lack of a reliable method for identifying proliferating cells. Although ‘gold standard’ methods involve the incorporation of thymidine analogues (e.g. [3H]thymidine or 5-bromodeoxyuridine) these methods are best performed prospectively. However, Spagnoli and colleagues used ex vivo [3H]thymidine labeling of human atherosclerotic tissue removed at time of peripheral artery surgery and determined that < 1% cells were proliferating [16]. Similarly, Leclerc and colleagues used ex vivo incorporation of 5-bromodeoxyuridine (BrDU) to demonstrate low levels of replication in RS human atherectomy specimens [17]. Immunodetection of cell cycle restricted proteins such as the PCNA or Ki-67 can also be used to measure replication. Unfortunately, all immunocytochemical methods of assessing replication are imperfect (e.g. detectable antigens may be masked by tissue fixatives or vary among different cell types). As well, differences in tissue fixation protocols and antibodies may have an important influence on antigen preservation and accessibility [18,19].

To a large extent, the current study was carried out in order to validate the results we previously obtained when we used PCNA immunolabeling to demonstrate infrequent and modest levels of proliferation in atherectomy tissue resected from RS coronary artery lesions [6]. These results were controversial and differed from those of Pickering et al. who found much higher levels of PCNA antigen expression in human atherectomy tissue (e.g. 15.2 ± 13.6% of all intimal cells for RS specimens) [11]. There are several methodological differences between these two studies that require discussion. In our immunocytochemistry protocol we used the monoclonal antibody PC-10 (DAKO, Carpenteria, CA) on tissue fixed in a methanol based fixative (methyl Carnoy’s solution). In contrast, Pickering and colleagues used a different tissue fixation protocol (e.g. 100% methanol) and a different monoclonal antibody to PCNA (Signet Laboratories, Dedham, MA). Second, there were differences in the criteria that were used to define immunopositive cells. Pickering and colleagues used human tonsil to optimize the PCNA immunolabeling procedure. The PCNA antigen is expressed for protracted intervals, therefore, the criteria used by these investigators may have lacked the specificity required to identify cells that are restricted to S phase of the cell cycle, and not G1, G2 or M phases. For example, these investigators also compared PCNA immunoreactivity and incorporation of [3H]thymidine in vascular smooth muscle cells in culture. Using their own criteria for PCNA immunolabeling they determined that, “Of 552 cells counted, 96% were PCNA positive but only 14% had incorporated [3H]thymidine into the nucleus”. In our previous study we defined positive immunolabeling with the PCNA antibody if the nuclei of epithelial cells in the crypts of small intestinal tissue immunolabeled dark black, as we knew from previous studies that only these cells routinely incorporated either BrDU or [3H]thymidine [5]. Hence, only atherectomy specimens cells with the entire nucleus labeled dark black were considered immunopositive.

Proliferation can also be assessed by detecting the expression of cell cycle-associated mRNA. Expression of some histone mRNA species (e.g. H3) is tightly linked to S phase of the cell cycle [12]. H3 mRNA is not polyadenylated, and is rapidly degraded when S-phase of the cell cycle is completed. Recently, three independent studies found an excellent correlation between the results of in situ hybridization for H3 mRNA and BrDU or [3H]thymidine labeling of paraffin embedded tissues [15,20,21]. PCNA mRNA expression is also cell cycle-associated. In the aforementioned study by Pickering and colleagues, in situ hybridization for the PCNA was also used to assess proliferation in RS coronary atherectomy specimens. These investigators found upregulated expression of PCNA mRNA in 7/11 PR and 11/11 RS specimens (including tissue from seven peripheral artery restenotic lesions). However, because PCNA mRNA expression is regulated by transcriptional as well as post-transcriptional mechanisms, expression of PCNA mRNA may not directly correlate with cell proliferation [22,23].

In the current study we used in situ hybridization to detect H3 mRNA expression and focused on coronary atherectomy specimens that were obtained in the early
Only 2 previous studies have used immunolabeling for the PCNA to identify cells if proliferation is present in the artery wall, and therefore, are more likely to contain proliferating cells if proliferation is present in the artery wall. The results of this study are comparable to those of our previous study using immunolabeling for the PCNA [6,13]. Only 2/17 coronary atherectomy specimens contained replicating cells, and both of the specimens had very low levels of replication (e.g. three and 19 replicating cells per slide). The tissue specimen with 19 replicating cells was obtained from a coronary lesion that had undergone a suboptimal angioplasty 1 day previously. In contrast, 9/12 directional atherectomy specimens from RS lesions of peripheral arteries contained replicating cells. Although the specimen with the highest replication profile (118 cells) was obtained from a peripheral artery lesion that had undergone a suboptimal atherectomy 2 days previously, high levels of replication were also found in lesions that had a remote history of a previous interventional procedure (e.g. 9 years).

Compared to PR lesions, RS peripheral arterial specimens more frequently contained proliferating cells. However, the percentage of cells that were proliferating in RS peripheral arterial specimens was low (e.g. ≤ 1.2% of all cells on a slide). Nonetheless proliferation indices were higher in the RS compared to PR peripheral arterial specimens (e.g. by as much as 100-fold). The apparent higher levels of proliferation in the RS peripheral specimens may be due to differences in the histomorphology of PR and RS lesions, possibly leading to the retrieval of tissue samples from different parts of the artery wall. For example, compared to RS specimens, PR lesion specimens may contain more fibrous cap and less cellular regions of the artery wall. Alternatively, proliferation may be more abundant in RS lesions, presumably due to events that were initiated with a previous interventional procedure. Why RS coronary artery specimens have proliferation profiles that are much lower than RS peripheral artery lesions is unknown. Given that directional atherectomy specimens from coronary arteries are much smaller than those obtained from peripheral arteries, it is possible that the lack of proliferation in coronary artery specimens may be due to the small sample size of these specimens. In other words, due to the smaller size of the directional coronary atherectomy specimens the likelihood of missing replication patches in the artery wall is increased. Alternatively, there may be size related factors that dictate the replication profile of lesions. For example, fewer cell doublings are required to result in obstructive lesions in a coronary artery compared to a larger femoral artery.

There are limitations to the current study. First, it is impossible to determine if the tissue that was resected from these procedures actually represents new arterial mass (as might arise with RS) or tissue that formed due to an underlying atherosclerotic process. This is particularly true for the specimens resected from lesions that had remotely been treated by an interventional procedure. Second, directional atherectomy specimens are primarily derived from the intima and media of diseased arteries and usually do not include adventitial tissue [26]. Recent data from large animal models of arterial injury and repair have focused attention on early proliferative events in the adventitia and more information is needed from human arteries [27,28]. Finally, due to the small size of these specimens, we were unable to find sufficient amounts of tissue to perform DNA nick-end labeling studies to assess apoptosis. Isner and colleagues demonstrated that the frequency of apoptosis is increased in RS compared to PR peripheral atherectomy specimens, and suggested that this was likely due to the higher replication profile of the RS specimens [29]. The data from this study support this concept.

In summary, these data demonstrate that replication commonly occurs in RS directional atherectomy tissue from human peripheral but not coronary artery lesions. Although the level of replication was low in all specimens, the frequency of replicating cells was higher in RS compared to PR lesion tissue obtained from peripheral arteries. In some instances, focal replication indices were remarkably high, and may represent patch expansion in the plaque. Therefore, while replication is more abundant in restenotic lesions, it does not appear to be a dominant process in the pathophysiology of RS. Nonetheless, because the duration of replication is unknown, the significance of protracted intervals of even low levels of proliferation cannot be discounted as an important factor in this disease process.

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