Highly Increased Cell Proliferation Activity in the Restenotic Hemodialysis Vascular Access After Percutaneous Transluminal Angioplasty: Implication in Prevention of Restenosis

Chi-Jen Chang, MD, Po-Jen Ko, MD, Lung-An Hsu, MD, Yu-Shien Ko, MD, PhD, Yu-Lin Ko, MD, PhD, Chin-Fen Chen, BSc, Chiu-Ching Huang, MD, Tsu-Shiu Hsu, MD, Ying-Shiung Lee, MD, and Jong-Hwei S. Pang, PhD

• ***Background:*** **The effect of percutaneous transluminal angioplasty (PTA) in the treatment of hemodialysis vascular access stenosis is attenuated by a high restenosis rate, which results mainly from neointimal hyperplasia. Cellular proliferation is one of the most important biological mechanisms involved in neointimal hyperplasia and may be a potential target of intervention to prevent restenosis.** ***Methods:*** **We investigated the activity of cellular proliferation of restenotic lesions by means of immunohistochemistry, using an antibody to the proliferating cell nuclear antigen. Specimens from 10 primary stenotic and 20 restenotic lesions of 30 Brescia-Cimino fistulae were obtained during revision.** ***Results:*** **The proliferation index of the restenotic group was strikingly significantly greater than that of the primary stenotic group (intima, $P < 0.001$; media, $P = 0.001$). Proliferation indices of patients with diabetes in the restenotic group were significantly higher than those of patients without diabetes (intima, $P = 0.028$; media, $P = 0.002$). In the restenotic group, proliferation indices correlated negatively with the interval from PTA to restenosis (intima, $r = -0.741$; $P < 0.001$; media, $r = -0.589$; $P = 0.006$) and positively with the number of PTAs per lesion (intima, $r = 0.754$; $P < 0.001$; media, $r = 0.506$; $P = 0.004$).** ***Conclusion:*** **We show markedly high cellular proliferation activity in early restenotic lesions of arteriovenous fistulae. These findings indicate that adjunctive antiproliferative therapy is mandatory in preventing restenosis after PTA, especially in patients with diabetes.** ***Am J Kidney Dis*** **43:74-84.**

INDEX WORDS: Hemodialysis (HD) vascular access; restenosis; neointima; cell proliferation.

HEMODIALYSIS access dysfunction remains the major morbidity in patients on maintenance hemodialysis therapy. Percutaneous transluminal angioplasty (PTA) is effective in treating these stenotic lesions. However, the benefit of this procedure is attenuated by a high restenotic rate of up to 36% to 62% within 6 months.[1-5] To keep arteriovenous fistulae (AVFs) in adequate function, repeated PTA within a short interval usually is inevitable. Both the primary and restenotic lesions are composed of hyperplastic neointimal tissues,[6,7] of which smooth muscle cell (SMC) proliferation is one of the major mechanisms in the pathogenesis. The cell proliferation rate among different vascular pathological states reported in previous studies varied from 0.5% to 20%.[8-16] Given the rapid progression rate and high incidence of restenosis of AVFs compared with other vascular beds in response to balloon angioplasty, more active cell proliferation may be involved in the pathogenesis. Although this knowledge is essential for the future development of an optimal strategy for the prevention of restenosis, it remains scarce.

Accordingly, this study investigated cell proliferation activity in restenotic lesions of efferent veins of Brescia-Cimino fistulae developing after PTA and compared it with that of primary stenotic lesions by analyzing the expression of proliferating cell nuclear antigen (PCNA) by means of an immunohistochemical method.

METHODS

Clinical Data

From November 2000 to July 2002, a total of 30 specimens of efferent veins of Brescia-Cimino fistulae, including 10 primary stenotic and 20 restenotic lesions developed after PTA, were obtained during surgical revision from 30 hemodialysis patients at Chung Gung Memorial Hospital, Taipei, Taiwan. All patients underwent revision because of severe stenosis with increased venous pressure and/or low flow volume during hemodialysis. The treatment of choice for stenotic hemodialysis access in daily practice at Chung

From the First Cardiovascular Division; and Departments of Cardiovascular Surgery and Nephrology, Chang Gung Memorial Hospital; and the Graduate Institute of Clinical Medical Sciences, Chang Gung University, Tao-Yuan, Taiwan.

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Address reprint requests to Chi-Jen Chang, MD, First Cardiovascular Division, Chang Gung Memorial Hospital, Fu-Shin Rd no. 5, Kwei-Shan, Tao-Yuan, Taiwan 333. E-mail: cchijen@adm.cgmh.org.tw

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Gung Memorial Hospital is PTA. Surgical revision was performed only when angioplasty failed; restenosis occurred soon after angioplasty, usually within 1 month; or patients refused angioplasty owing to frequent restenosis or other reasons. During the PTA procedure, high-pressure dilation of lesions exceeding 12 atmospheres was performed frequently to achieve residual stenosis less than 30%. Moreover, balloon inflation of up to 18 atmospheres also was performed sometimes. We excluded specimens retrieved from polytetrafluoroethylene graft interposed fistulae to avoid heterogeneity in biological natures. The diagnosis of stenosis was confirmed by duplex ultrasound in all cases.

Specimens were rinsed briefly with normal saline and fixed in 10% formalin immediately after harvesting. Tissues then were embedded in paraffin, processed according to conventional technique, and cut into 5-μm thick sections.

Immunohistochemistry

Serial sections from each specimen were reacted with anti-PCNA antibody (PC10; Neomarkers, Fremont, CA; 1:200 dilution) to identify proliferating cells and anti–smooth muscle α-actin (Muscle actin clone 1A4; Sigma, St Louis, MO; 1:100 dilution), anti-CD31 (JC/70A; NeoMarkers; 1:100 dilution), or anti-CD68 (KP1; Neomarkers; 1:100) antibodies as cell markers for vascular SMCs, endothelial cells, and macrophages, respectively. Anti-PCNA immunoreaction was performed repeatedly with good reproducibility, and the 1:200 dilution provided the best signal-to-background staining ratio. Venous tissues obtained from patients with end-stage renal disease during AVF creation and human intestinal tissues were used as negative and positive controls in staining sessions, respectively.

Single-labeling immunohistochemistry. First, sections were deparaffinized with xylene and rehydrated in graded ethanol series. Next, sections were microwave heated for 10 minutes in a 0.01-mol/L citrate buffer solution (pH 6.6). After blocking endogenous peroxidase activity with 3% hydrogen peroxide, primary antibodies were applied for 60 minutes at room temperature, except for anti-PCNA antibody, which was applied overnight at 4°C. A biotinylated antimouse secondary antibody then was applied for 30 minutes, followed by a 10-minute incubation with peroxidase-conjugated streptavidin (Dako, Carpinteria, CA; 1:200 dilution). Slides were exposed to 3, 3′ diaminobenzidine (DAB) to obtain a brown reaction product and counterstained with contrast blue.

Double-labeling immunohistochemistry. Double-labeling immunohistochemistry was performed on tissue sections adjacent to the section used for PCNA staining. PCNA immunostaining was performed first as described. Sections were washed copiously with phosphate-buffered saline and exposed to the second primary antibodies for 60 minutes. Then the Dako EnVision doublestain kit (K 1395; Dako) was applied. Finally, the alkaline phosphatase–labeled polymer was applied for 30 minutes and developed with the alkaline phosphatase substrate, which produced a red reaction product.

Cell Counting and PCNA Index

The number of total cells and PCNA-positive cells per section was counted manually under a light microscope at original magnification $\times$400 under an ocular grid by 2 investigators blinded to clinical details. Cells with nuclei labeled brown or dark brown were recognized as PCNA positive. Because heterogeneity in the distribution of PCNA-positive cells might cause sampling bias, cell counting was performed to cover most fields of each section. The number of PCNA-positive macrophages or endothelial cells was counted on the adjacent sections used for double staining. Total cell counts of these adjacent sections were assumed to be equal. PCNA index is defined as the number of PCNA-positive cells divided by the total number of cells.

Statistical Analysis

Data are expressed as mean $\pm$ SD. Owing to the small number of observations in each group, nonparametric statistical analysis was conducted. When comparing continuous data among independent groups, Mann-Whitney test was used. Spearman's correlation coefficient was computed to examine the linear relation between 2 continuous variables. All P presented are 2-sided, and the level of significance is 0.05.

RESULTS

Clinical Characteristics

Clinical characteristics are listed in Table 1. In the restenotic group, the number of PTAs per lesion ranged from 1 to 6, and the interval from last PTA to revision ranged from 8 hours to 16 months (4.5 $\pm$ 5.0 months). Because mechanisms of restenosis potentially may differ between lesions with restenosis that occurred relatively early after PTA and those that occurred relatively late, we divided the restenotic group into the early and late restenotic subgroups for additional analysis. Fifteen of 20 patients in the restenotic group with an interval less than 6 months were defined as the early restenostic subgroup, and the remaining 5 patients with an interval longer than 6 months, as the late restenostic subgroup. In the restenotic group, the underlying cause of renal failure was diabetes in 5 of 20 patients; all belonged to the early restenotic subgroup.

General Observations

Intimal layers of all specimens in both the primary stenotic and restenotic groups were composed of neointimal tissues, which encroach most of the luminal space (Fig 1A and B). In some specimens, lumens were nearly occluded. Two histological appearances of the neointima could

Table 1. Clinical Characteristics of Study Subjects

Patient No.	Age (y)	Sex	Duration of ESRD (mo)	Life of Access (mo)	No. of PTAs	Interval Between PTA and Revision (mo)	Cause of ESRD
Primary stenosis							
1	18	M	6	6	0	—	Chronic glomerulonephritis
2	51	F	16	16	0	—	Unknown
3	47	F	3	3	0	—	Unknown
4	65	M	14	14	0	—	Renal artery stenosis
5	52	M	28	28	0	—	Unknown
6	69	M	12	12	0	—	Unknown
7	69	F	2	2	0	—	Unknown
8	22	F	24	24	0	—	Chronic glomerulonephritis
9	64	M	2	2	0	—	Unknown
10	71	M	2	2	0	—	Unknown
Restenosis							
11	46	M	14	14	3	0.75	Diabetes
12	35	F	21	21	6	2.75	Chronic glomerulonephritis
13	69	F	7	7	2	2.25	Unknown
14	56	M	6	6	1	1.75	Unknown
15	32	F	92	43	3	1	Chronic glomerulonephritis
16	83	F	18	18	1	3	Hypertension
17	51	F	22	22	5	2	Bladder cancer
18	63	M	18	18	1	0.5	Diabetes
19	61	M	17	17	2	0.5	Diabetes
20	65	M	12	12	1	0.75	Diabetes
21	73	F	26	26	5	6	Unknown
22	67	F	8	8	1	5.5	Unknown
23	74	F	7	7	3	0*	Unknown
24	57	M	3	3	1	0.5	Diabetes
25	55	F	72	72	1	2	Unknown
26	73	M	16	16	1	9	Unknown
27	66	F	36	36	2	16	Unknown
28	80	F	26	26	1	9	Hypertension
29	52	M	84	84	1	15.5	Chronic glomerulonephritis
30	68	M	72	72	1	11	Polycystic kidney disease

Abbreviation: ESRD, end-stage renal disease.
*The interval was 8 hours.

be identified. The first was characterized by SMCs that were variably oriented in loose extracellular matrix. The second was characterized by a high density of SMCs that were partially aligned with one another. Both morphological types often coexisted in neointima of 1 tissue section. Similar morphological patterns also have been observed in neointimal lesions of the arterial tissues by Glagov.[17] Notably, distribution of both morphological types generally did not differ between the primary stenotic and restenotic lesions. In the restenotic group, disruption of the media, defined by loss of continuity of the medial SMC layer (Fig 1C), was noted in 12 of 15 samples in which an entire cross-sectional area of vessel could be obtained to examine the continuity of the media. In most specimens, neovascularization was common in intimal and medial layers.

Cell Proliferation

PCNA indices of venous tissues obtained during AVF creation were less than 1% in different runs of immunohistochemistry, which served properly as a negative control (Fig 2B). PCNA indices of intestinal tissues were constant, ranging from 22% to 28% in different runs of the immunostaining procedure (Fig 2A). This index was similar to that found in a previous study and could be used properly as a positive control.[13] Both primary stenotic and restenotic lesions showed active cell proliferation in the neointima

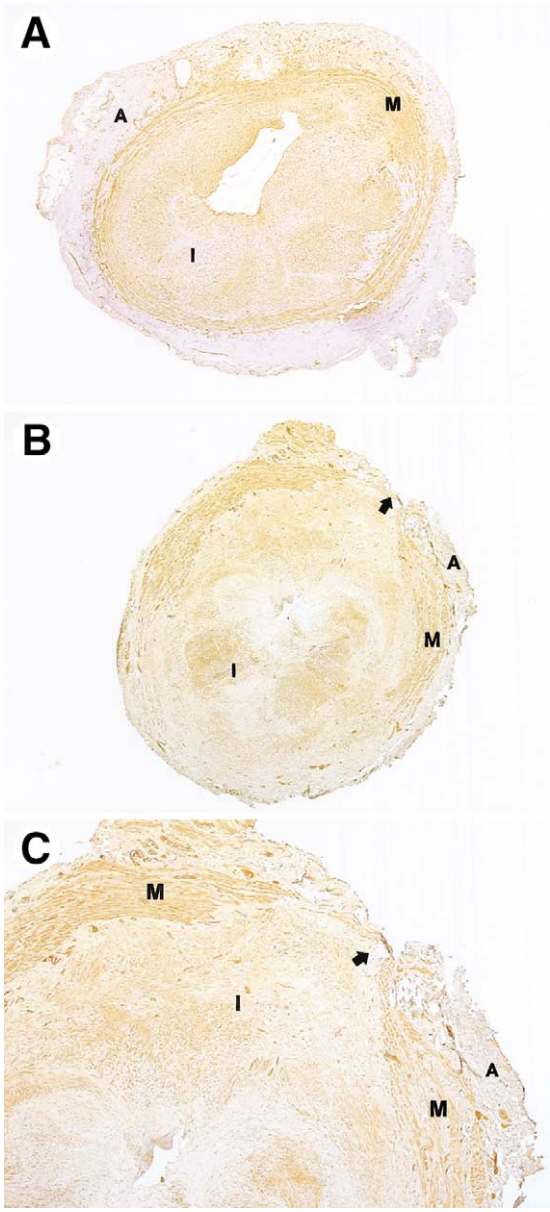


Fig 1. Representative photomicrographs of (A) primary stenotic and (B) restenotic lesions of human AVFs for hemodialysis access stained with anti-smooth muscle α-actin antibody. Lumens of both lesions were encroached by hypercellular neointimal tissues, in which most cells were smooth muscle α-actin positive. (Original magnification ×20.) (C) Greater magnification of the restenotic lesion shows disruption (arrow) of the medial layer resulting from PTA. Abbreviations: I, intima; M, media; A, adventitia. (Original magnification ×40.)

and media (Fig 2C and D). No obvious difference in the distribution pattern of PCNA-positive cells was noted between the 2 groups.

Figure 3 shows the PCNA index of each lesion in the primary stenotic and restenotic groups. PCNA indices of the restenotic group were markedly higher than those of the primary stenotic group (intima, $P < 0.001$; media, $P = 0.001$). Comparing PCNA indices of 2 restenotic subgroups separately with that of the primary stenotic group showed the difference was even more remarkable between the early restenotic and primary stenotic groups (intima, $P < 0.001$; media, $P < 0.001$). In the late restenotic subgroup, the PCNA index of the intima remained slightly higher than that of the primary stenotic group ($P = 0.017$). However, the index of the media of this subgroup did not differ from that of the primary stenotic group ($P = 0.244$). When PCNA indices of 2 restenotic subgroups were compared with each other, the indices were markedly higher in the early restenotic than late restenotic subgroup (intima, $P = 0.004$; media, $P = 0.010$).

In the primary stenotic group, we also tested whether cell proliferation was more active in lesions with stenosis occurring within 6 months after AVF creation than those occurring later than 6 months. Stenosis occurred within 6 months in 5 of 10 lesions and later than 6 months in the remaining 5 lesions. Proliferative indices of the early and late groups were 19% ± 4% versus 18% ± 5% ($P = 0.84$) in intima and 18% ± 5% versus 14% ± 11% ($P = 0.22$) in media. In contrast to the restenotic group, we did not find higher proliferative indices in stenotic lesions that occurred early after AVF creation.

Because diabetes is a well-established risk factor in various vascular diseases and also has been reported to be an independent predictor of AVF failure,[18] we analyzed PCNA indices of patients with and without diabetes in the restenotic group. The result showed significantly higher PCNA indices in the diabetic group (intima, $P = 0.028$; media, $P = 0.002$). In an animal model of angioplasty, SMC proliferation was proportional to degree of vascular injury.[19,20] Therefore, we also compared the proliferation index between those with and without medial rupture. The result showed no significant difference between the 2 groups (52% ± 19% versus 42% ± 21%; $P = 0.52$ for

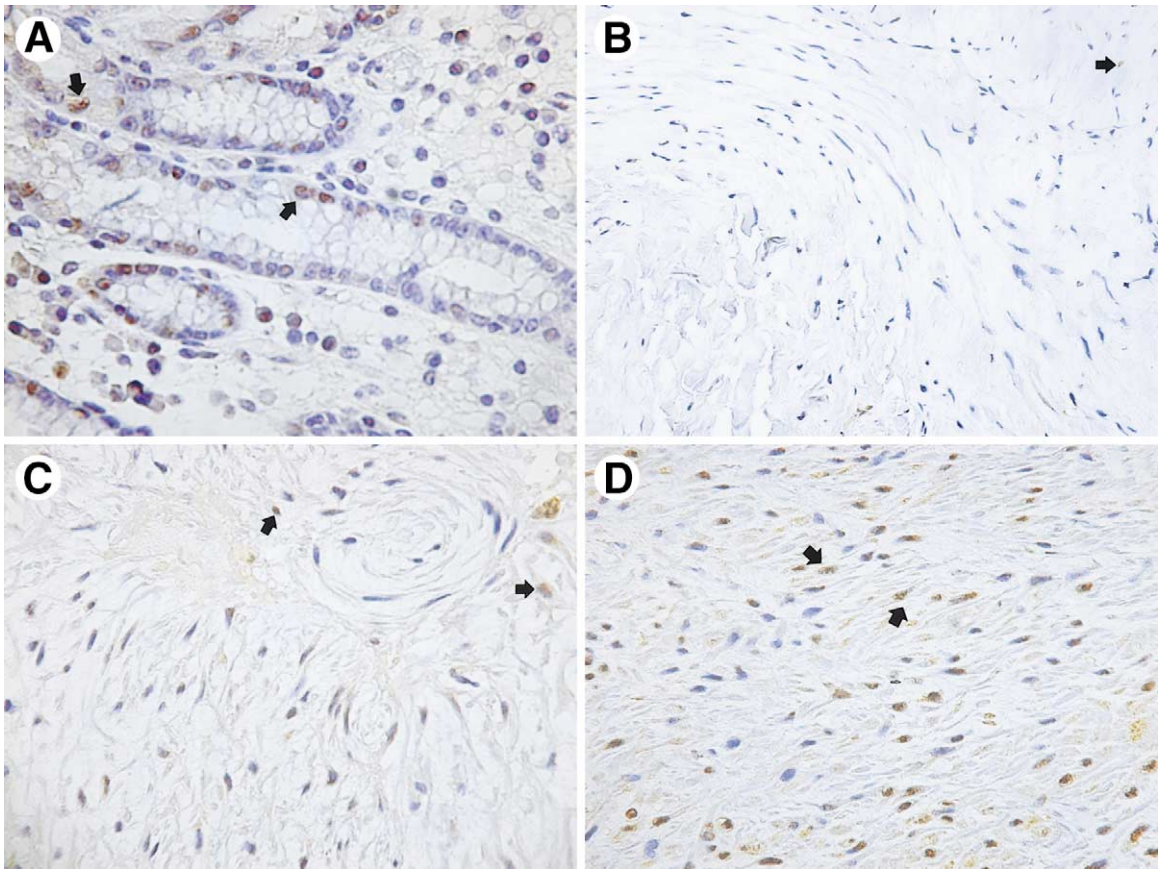


Fig 2. Representative photomicrographs of PCNA immunoreactivity of the hemodialysis vascular access. (A) Human intestinal tissue was used as the positive control. Note nuclei of proliferative crypt epithelium are labeled dark brown (arrows). (B) Venous tissue obtained during creation of vascular access. Note very few nuclei are positively labeled (arrow). (C) Neointima of a primary stenotic lesion shows several PCNA-positive nuclei (arrows). (D) Neointima of an early restenotic lesion of an AVF shows abundant PCNA-positive nuclei (arrows). (Original magnification ×400.)

intima; 32% ± 22% versus 42% ± 16%; $P = 0.45$ for media).

In the restenotic group, there was a strongly negative correlation between PCNA index and interval from PTA to restenosis (intima, $r = -0.741$; $P < 0.001$; media, $r = -0.589$; $P = 0.006$), as shown in Fig 4. Moreover, PCNA indices of the intima strongly correlated positively with number of PTAs per lesions ($r = 0.754$; $P < 0.001$). However, the correlation between PCNA indices of the media and number of PTAs per lesion was much weaker despite remaining statistically significant ($r = 0.506$; $P = 0.04$; Fig 5).

We also performed double-labeling immnunohistochemical studies using anti-PCNA and cell type–specific antibodies to identify the cell type of PCNA-positive cells (Fig 6). The SMC, identified by positive anti-SMC α-actin immunostaining, was the predominant cell type that expressed PCNA. In both the primary and restenotic groups, PCNA-positive endothelial cells or macrophages were scarce, identified by positive anti-CD31 or anti-CD68 immunostaining, respectively. In the primary stenotic group, endothelial cells and macrophages comprised 3% ± 2% and less than 1% of PCNA-positive cells in the intima and 5% ± 3% and less than 1% in the media, respectively. In the restenotic group, endothelial cells and macrophages comprised 8% ± 4% and 5% ± 5% of PCNA-positive cells in the intima and 10% ± 6% and 8% ± 5% in the media, respectively.

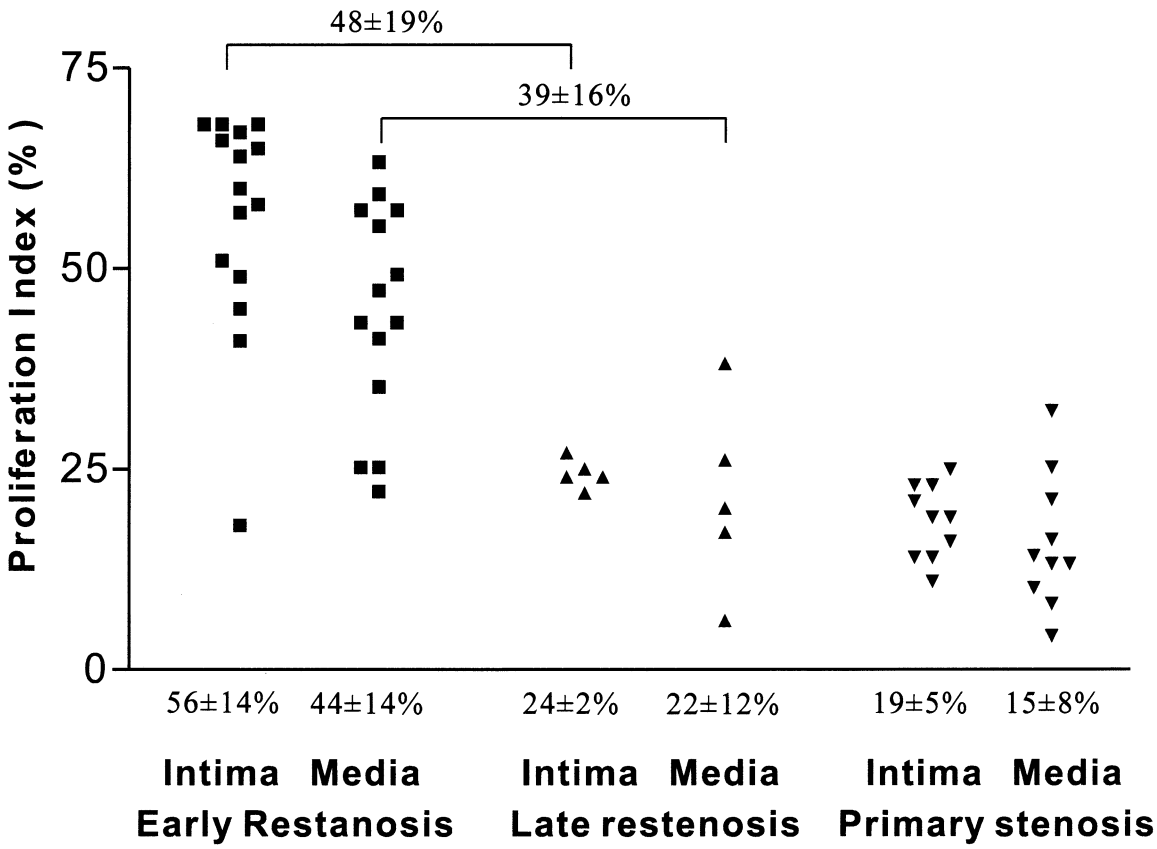
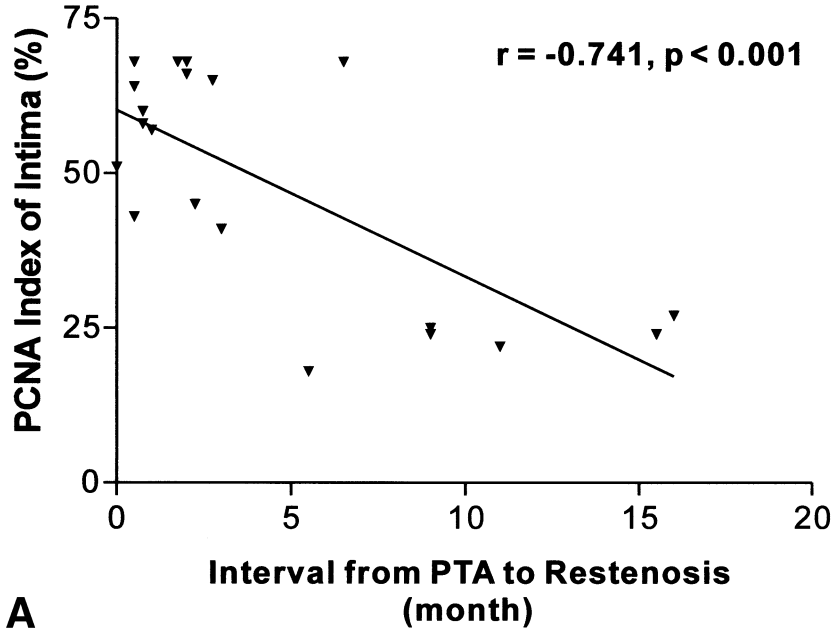


Fig 3. Proliferation indices per specimens in the primary stenotic and restenotic groups. The latter was divided further into the early restenotic (≤6 months) and late restenotic (>6 months) subgroups.

DISCUSSION

The present study shows markedly increased cell proliferation activity in post-PTA restenotic lesions of hemodialysis vascular access, particularly in diabetic and early restenotic lesions. Cell proliferation activity was much higher compared with that of primary stenotic lesions, although both lesions showed similar histological changes. Cell proliferation activity of early restenotic lesions in the present study was much higher than any of those previously reported in other diseased human vascular tissues.[8-15] This extremely high cell proliferation activity is consistent with the rapid progression rate and very high incidence of restenosis of AVFs after PTA. This study also showed that the greater proliferation rate strongly correlated negatively with a shorter interval from PTA to restenosis, suggesting that proliferation activity is a critical determinant of progression rate of restenosis. These findings together suggest that cell proliferation, predominantly of SMCs, has an important role in the pathogenesis of post-PTA restenosis of AVFs. In primary stenotic lesions, although proliferation activity was lower than that in restenotic lesions, it remained higher compared with most other diseased vessels previously reported. In contrast to the restenotic group, we did not find a higher proliferative index in primary stenotic lesions that occurred early after AVF creation compared with those that occurred later. This finding suggests that markedly high cell proliferation activity is a relatively unique response to PTA, but not to AVF creation.

PCNA expression determined by immunohistochemistry commonly is used in estimating cell proliferation in vascular tissues. Gorden et al[15] compared this method in paraffin-embedded samples with in vivo thymidine labeling, and good correlation between thymidine labeling and PCNA immunostaining was shown. However, a much greater rate of PCNA immunohistochemi-



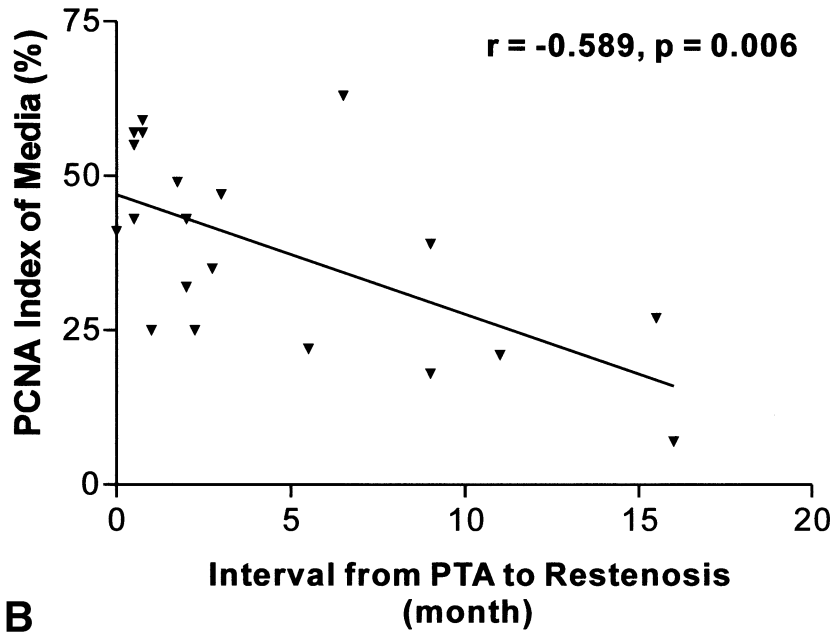


Fig 4. Relationship between proliferation indices of the (A) intima and (B) media in the restenotic group and interval from PTA to restenosis. The higher proliferation indices in both intima and media are associated with a shorter interval from PTA to restenosis.

cal labeling compared with thymidine labeling was noted, which likely is caused by the relatively longer half-life of PCNA protein in cells. The extremely high PCNA index shown in restenotic lesions was unusual in vascular tissue. However, very low PCNA indices of less than 1% in venous tissues obtained before AVF creation, serving as the negative control, ruled out the nonspecific reaction of PCNA immunostaining. In addition, PCNA indices of intestinal tissues and primary stenotic lesions shown in this study were similar to those reported in previous studies,[14,15] which further ensured the specificity of the immunostaining procedure.

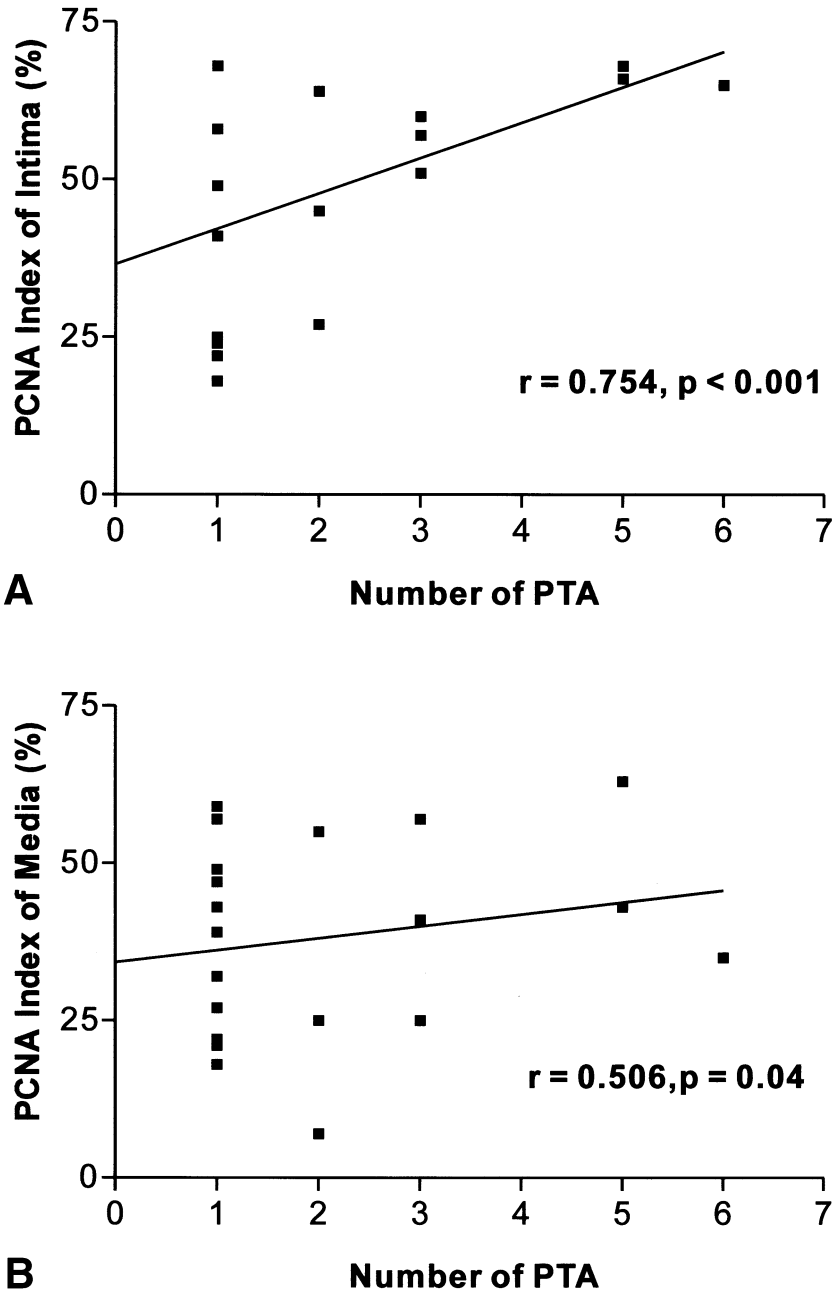


Fig 5. Relationship between proliferation indices of the (A) intima and (B) media in the restenotic group and total number of PTAs to restenosis. Higher numbers of PTAs are associated with greater proliferation indices in both intima and media.

Cell proliferation is one of the most important cellular events involved in vascular pathogenesis, which usually results in narrowing of vascular lumen and thus compromises blood flow. However, cell proliferation rates in different diseased vessels, determined by the expression of PCNA protein or messenger RNA, varied from as low as 0.5% to up to 24.6%.[8-16] One essential issue that must be clarified when analyzing these variable data is whether the pathological change in vascular lesions is atherosclerosis or neointimal hyperplasia. In atherosclerotic lesions, such as primary stenosis of the coronary artery, the PCNA expression rate was relatively low, ranging from 0% to 7.2% in different reports.[10,12,13,15] Conversely, neointimal hyperplastic tissues, such as the stenotic venous anastomosis of polytetrafluoroethylene graft hemodialysis access and re-

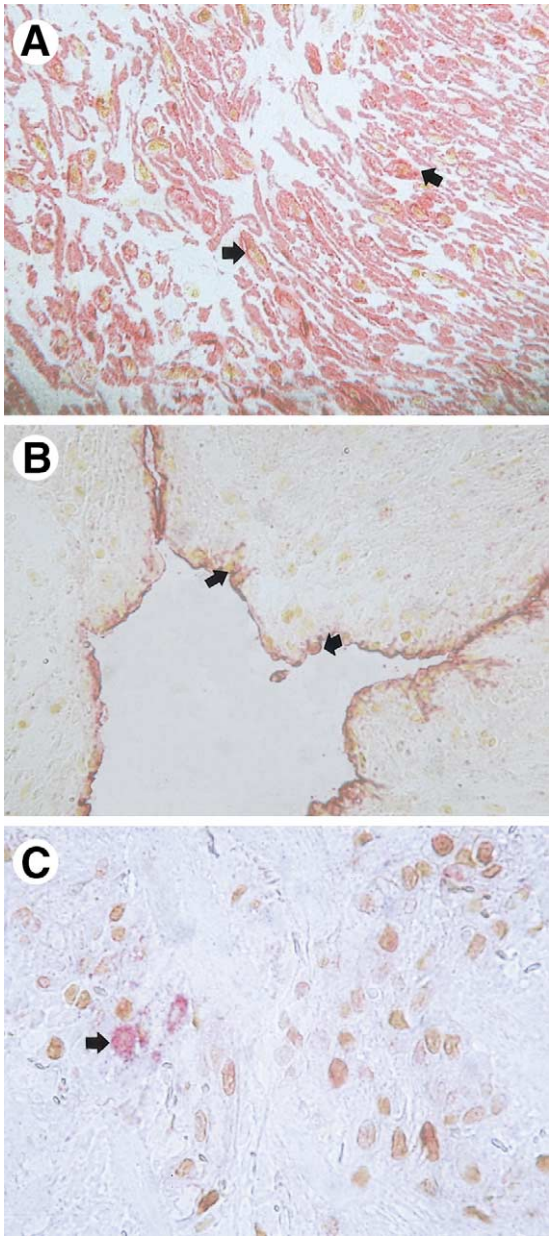


Fig 6. Double-label immunocytochemistry of stenotic specimens of vascular access for hemodialysis showing PCNA-positive nuclei, labeled brown, and cytoplasm of cells identified by a cell-specific antibody, in red. (A) Anti-PCNA and anti–smooth muscle α-actin labeling of proliferating SMCs (arrows) in an early restenotic lesion. Note a very high proportion of SMCs are proliferating. (B) Anti-PCNA and anti-CD31 labeling of proliferating endothelial cells (arrows). Many luminal endothelial cells are proliferating. (C) Anti-PCNA and anti-CD68 labeling of proliferating macrophages (arrow) in a restenotic lesion. Note 1 proliferating macrophage in the media close to the neovasculare. (Original magnification ×400.)

stenotic coronary or peripheral arterial lesions that developed after percutaneous angioplasty or stenting, had much greater PCNA expression, ranging from 18% to 24% in different layers of the vessel wall.[11,14,16] These data closely resemble our findings for primary stenotic AVFs, which were composed exclusively of neointimal tissue.

To the best of our knowledge, this study is the first to investigate cell proliferation activity in post-PTA restenotic neointimal tissue of human AVFs. The underlying mechanism for the remarkably high cell proliferation activity remains to be investigated. However, there are several potential explanations. Neointimal hyperplasia is the exclusive pathological change in restenotic lesions examined in this study, just as in primary stenotic lesions. However, these were additional neointimal hyperplasia developed on the base of preexisting neointimal tissue in response to balloon dilation. Cells in the preexisting neointimal tissue may have acquired greater proliferation potential and therefore responded quickly and actively to the stimulation of PTA.

Additionally, the unique hemodynamic pattern of AVFs may be another potential cause of the high cell proliferation activity. Liu[21] showed that eddy flow occurring in the vein graft potentiates proliferation activity of focal neointimal lesions mediated by angiotensin II type I receptor. This unique flow pattern could exert an additional effect in enhancing proliferation activity in lesions of AVFs stimulated by PTA.

The poor integrity of internal elastic laminae of the venous wall compared with those of the arterial wall and the severe injury of vessel wall resulting from high-pressure balloon dilation were 2 other potential contributors to the high proliferation activity in restenotic lesions. Indolfi et al[19,20] recently showed a strong association between severity of injury of vessel wall and proliferation rate of vascular SMCs in a rat carotid balloon dilation model. Medial disruption was noted in 12 of 15 restenostic lesions valid for investigation in this study. However, restenotic lesions with medial rupture were not associated with a higher proliferation index.

The most important clinical implication of the findings in this study is that suppression of cell proliferation induced by balloon angioplasty is essential to reduce the restenotic rate of vascular

access after PTA, particularly for patients with diabetes. For prevention of primary stenosis, antiproliferation also may be helpful because cell proliferation activity in primary lesions, although not as high as that in restenotic lesions, was much higher than that in most other pathological vascular tissues reported. Given the markedly active cell proliferation compared with other vascular pathological characteristics, more aggressive adjunctive therapy is reasonable, such as radiation therapy or local high-dose antiproliferative drug therapy. Brachytherapy in combination with PTCA has been proven to be the only effective treatment in reducing the restenosis rate of diffuse in-stent restenotic coronary lesions,[22] which comprise typical neointima, as are AVF lesions. This finding supports that effective antiproliferation therapy is mandatory in preventing restenosis after balloon angioplasty for lesions composed of neointimal hyperplasia.

Animal studies using a porcine model also showed that irradiation therapy after AVF creation significantly reduced intimal formation.[23,24] This hypothesis should be tested by clinical trials with a tailored dose of radiation. Additionally, the superficial localization of the hemodialysis access makes it possible to deliver antiproliferation drugs percutaneously and repeatedly, which may provide another potential modality to prevent restenosis. Drugs effective in inhibiting SMC proliferation, especially dipyridamole, shown in clinical trials to reduce graft thrombosis, possibly by inhibiting neointimal hyperplasia,[25,26] may have greater efficacy in reducing restenosis if administered percutaneously in a higher dose.

In conclusion, this study verifies that active cell proliferation is one of the most important mechanisms in restenosis of hemodialysis vascular access after PTA, particularly in patients with diabetes. Proliferation activity was much greater than in any other vascular bed reported. These findings indicate that effective inhibition of cell proliferation is required to prevent restenosis in the vascular access after PTA.

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