Presence of bone-marrow- and neural-crest-derived cells in intimal hyperplasia at the time of clinical in-stent restenosis

Dirk Skowasch, Alexander Jabs, René Andrie, Sabine Dinkelbach, Berndt Lüderitz, Gerhard Bauriedel

Department of Cardiology, Heart Center University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany

Received 17 April 2003; received in revised form 3 August 2003; accepted 3 September 2003

Abstract

Objective: Intralesional data of coronary target lesions following stent implantation are infrequent. In addition, there is ongoing controversy on the origin of neointimal cells. In this respect, several lines of evidence revealed bone-marrow-derived endothelial progenitor and dendritic cells (DCs) as well as neural-crest-derived cells (NCCs) to contribute to atherosclerosis. Therefore, the objective of the present study was to assess cellularity, cell type and origin of neointimal cells in in-stent restenosis (ISR).

Methods: Atherectomy specimens from 17 patients with coronary in-stent restenosis (n=10; time post-stenting ≤ 3 months) and with peripheral in-stent restenosis (n=7; 7 ≤ 3 months) versus those from 10 patients with primary lesions were immunohistochemically examined for the presence of the determinants CD34, AC133, S100, glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), nerve growth factor receptor (NGFR) and α-smooth muscle actin followed by computer-assisted morphometry.

Results: In-stent restenosis probes consistently demonstrated homogeneous hypercellularity (942 ± 318 cells/mm²) compared to de novo lesions (347 ± 120 cells/mm², P<0.001). α-smooth muscle actin positive cells occupied 67% of intimal cells in in-stent restenosis. As a key finding, expression of endothelial progenitor cells (CD34: 7.1 ± 2.5% positive/total cells vs. 0.6 ± 0.7%, P<0.001; AC133: 7.0 ± 3.4% vs. 1.0 ± 0.7%, P<0.001), dendritic cells (S100: 9.8 ± 5.6% vs. 1.4 ± 1.1%, P<0.001) and neural-crest-derived cells (GFAP: 7.9 ± 2.4% vs. 3.1 ± 1.0%; NSE: 4.4 ± 2.6% vs. 1.3 ± 1.6%; NGFR: 4.2 ± 2.5% vs. 1.1 ± 0.7%; each P<0.001) was significantly increased in in-stent restenosis compared to primary lesions.

Conclusions: Bone-marrow- and neural-crest-derived cells, the most dendritic cells, are consistently present in in-stent restenosis, whereas α-smooth muscle actin positive cells constitute the largest intimal cell pool. Our data suggest the recruitment of primarily extravascular cells within neointima formation in human in-stent restenosis.

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Keywords: Atherosclerosis; Histopathology; Restenosis; Stem cells; Stents

1. Introduction

In-stent restenosis (ISR) is an ongoing major limitation following stent implantation. The pathobiological events underlying ISR are still poorly understood [1]. Several animal studies, but only a few human studies, report on the intralesional changes after stent implantation, underscoring migration and proliferation of smooth muscle cells (SMCs) as central mechanisms [2,3]. Although it is widely accepted that the main component of chronic neointima is formed by α-smooth muscle (SM) actin positive cells, there is ongoing controversy on the origin of neointimal cells. In particular, it is unclear whether these cells derive from the arterial wall, from extravascular sources or from circulatory precursor cells [2,4–8]. As yet, a significant number of intimal mesenchymal cells remain unidentified despite a large body of previous work.

Several lines of evidence recently revealed the presence of pluripotent mesenchymal cells originating from primarily extravascular reservoirs such as the bone marrow. Likewise, few reports showed rapid, transient mobilization of endothelial precursor cells (EPCs) after vascular trauma [9] and the presence of bone-marrow-derived cells in solid neointima [8] or in lesions after allografting [10]. Moreover, bone-marrow-derived dendritic cells (DCs) were detected in arterial lesions, putatively contributing to T-cell activation.
and progression of atherosclerosis [11–13]. In addition, neural-crest-derived cells (NCCs) can differentiate into SMCs and are also found in the wall of cardinal veins [14]. Therefore, we hypothesized that EPCs, DCs and NCCs may be involved in neointima formation following stent implantation. The objective of the present study was to assess the presence of smooth muscle cells, endothelial progenitor cells, dendritic cells and cells exhibiting neural-crest markers in atherectomy specimens obtained from patients with clinical ISR.

2. Methods

2.1. Patients and arterial specimens

A series of 27 symptomatic patients (21 men and 6 women; mean age, 58 ± 11 years) had been treated by percutaneous directional coronary atherectomy (DCA; Danmed, Isernhagen, Germany) [15,16]. Table 1 summarizes patient and lesion characteristics. Atherectomy specimens were obtained from 20 coronary and 7 peripheral arteries, including 10 primary and 17 in-stent restenotic lesions. All patients received heparin intravenously 30–60 min before and then during the DCA procedure. Before the percutaneous treatment, all patients had given their informed consent for subsequent analysis of the plaque tissue, and the local ethics committee had approved the study. The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovasc. Res. 1997; 35: 2–4).

2.2. Immunohistochemistry

The removed tissue was fixed in 4.5% buffered formaldehyde and embedded in paraffin. Serial thin sections of 4 μm were cut from these segments for immunohistochemical analysis. First, the paraffin-embedded tissue was dewaxed and rehydrated. Then, the tissue was incubated twice with citrate buffer in microwave. After cooling down for 15 min at room temperature, the tissue segments were incubated for 30 min with rabbit serum 1:5 (Dianova, Hamburg, Germany), to block nonspecific binding sites. The next step was to add either the monoclonal antibodies, anti-α-smooth muscle actin (clone 1A4, Sigma, Deisenhofen, Germany, 1:250), anti-CD34 (clone 581, Dianova, 1:500), anti-AC133 (clone AC133/1, Miltenyi Biotec, Bergisch-Gladbach, Germany, 1:200), anti-neuron-specific enolase (NSE) (clone BB5/NC/VI-H14, Dako, 1:400) and anti-nerve growth factor receptor (NGFR) (clone NGFR 51, Dako, 1:100) or the polyclonal rabbit anti-S100 (Sigma, catalog number S2644; 1:500) and anti-glial fibrillary acidic protein (GFAP) (Dako, Hamburg, Germany, code number Z 0334; 1:500). For polyclonal antibodies, AffiniPure mouse anti-rabbit IgG (Dianova, code number 211-005-109) was applied for 30 min at room temperature following primary antibody incubation. The color reaction was done with APAAP marking (Dianova) according to a standard protocol. The color substrate of alkaline phosphatase was fast red (Sigma). Finally, nuclear counterstaining was done with hematoxylin.
Double staining experiments were performed with CD34/α-actin, AC133/α-actin, S100/α-actin, GFAP/α-actin, NSE/α-actin, S100/CD34, S100/AC133, CD34/AC133, GFAP/CD34, GFAP/AC133, AC133/NSE and GFAP/S100 according to an avidin–biotin standard protocol. After inhibition of endogenous peroxidase activity with methanol and hydrogen peroxide (3%), the tissue sections were incubated twice in citrate buffer in a microwave oven. After cooling down, unspecific binding sites were blocked by normal goat serum 1:10 (Dianova). The sections were incubated with the first antibody (CD34 1:200, AC133 1:150, S100 1:100, GFAP 1:500, NSE 1:400) for 40 min at 37 °C, respectively. For polyclonal S100 and GFAP, AffiniPure mouse antirabbit IgG was subsequently applied as described above. Antibody binding was visualized with a biotinylated goat anti-mouse antibody (1:50, Vector, Grünberg, Germany), followed by the ABC complex (Vector) and aminoethylcarbazole (ICN, Eschwege, Germany) as chromogen. Consecutively, sections were incubated with either CD34, AC133, S100, NSE or α-actin antibodies for 40 min at 37 °C. These bound antibodies were detected with horse serum (1:10, Dianova) and a biotinylated horse anti-mouse antibody (1:100, Vector) as

![Fig. 1](https://academic.oup.com/cardiovascres/article-abstract/60/3/684/336750)

**Fig. 1.** (a) and (c) Representative photomicrographs of in-stent restenosis (ISR) tissue obtained from the left anterior descending coronary artery of a 58-year-old man 6 months after stent implantation. The hypercellular ISR lesion shows high percentage of CD34+ and AC133+ cells indicating bone-marrow origin; see the typical morphological features of dendritic cells (DCs) with long cytoplasmic processes (arrow). (b and d) Note that only a few signals of CD34 and AC133 were observed in primary lesions. (e) Strong cell-bound S100 signalling in the same patient as in (a) and (c) demonstrating the presence of DCs. (f) DCs are found sparsely distributed in a primary coronary lesion; bar = 50 μm.
described above and VIP (Vector) as chromogen. Washing steps between incubations were performed in phosphate-buffered saline (pH 7.3).

Negative controls included omission of the antibodies, staining with unspecific antibody (mouse monoclonal IgG1, clone NCG01, Dianova and ChromPure rabbit IgG, code number 011-000-003, Dianova, respectively) in the same concentrations as the primary antibodies as well as tissue samples taken from non-implanted saphenous vein grafts and normal mammary arteries.

2.3. Morphometric assessment of cell density and expression

Hematoxylin-stained histological sections allowed the detection of cellular nuclei. Assessment of cell density was performed by a computer-assisted morphometry system (VFG 1 graphic card/VIBAM 0.0. software) to count stained cell nuclei per area (0.04 mm²) and to calculate the final cell density [17,18]. Pictures were obtained with an Optiphot-2 microscope (Nikon, Düsseldorf, Germany) and downstream KP-C 553 CCD video camera (Hitachi, Rodgau, Germany). Five randomly selected areas, each encompassing 0.04 mm², were assessed per tissue sample. The morphometric data were evaluated by two independent examiners. The percentage of positive cells was determined as the number of positive cells per total number of cells for each lesion, as recently described [17].

2.4. Statistical analysis

Data are presented as the percentage (mean ± S.D.) of positively stained cells among five randomly chosen high-power fields. We assessed group differences by use of χ² test for categorical variables. Probability was calculated with Mann–Whitney U-test for differences in cell density and expression of intimal determinants. P values of <0.05 were considered to be statistically significant.

3. Results

The present report studies a collective of 17 in-stent restenosis (ISR) versus 10 primary lesions from patients presenting with stable angina. Patient characteristics, such as age, gender and medication (Table 1), did not differ between ISR and primary lesions. Comparison of cardiovascular risk factors revealed a higher prevalence only for diabetes in the ISR group (P=0.03), but not for hypertension, hyperlipidemia, familial disposition, nicotine or obesity (Table 1). Representative examples of ISR lesions that demonstrate the presence of selected key determinants are illustrated in Fig. 1. Quantitative data resulting from pathological examination of ISR probes are summarized in Fig. 2. Consistently, ISR tissue from all 17 patients showed marked cellularity that was higher in ISR com-

![Fig. 2. Expression of studied determinants in 17 ISR (■) versus 10 primary lesions (□). Asterisks indicate statistical significance (P<0.001).](https://academic.oup.com/cardiovascres/article-abstract/60/3/684/336750)
pared to de novo lesions (942 ± 318 vs. 347 ± 120 cells/mm², \( P<0.001 \)). There was no significant difference between cellularity in ISR tissue from coronary or peripheral vascular bed (998 ± 276 vs. 863 ± 379 cells/mm², \( P=0.38 \)). Although α-smooth muscle (SM) actin positive cells comprised the largest cell population in both ISR and primary lesions, ISR lesions contained more α-SM actin positive cells than primary lesions (67.4 ± 15.2% vs. 33.7 ± 15.5%, \( P=0.04 \)).

Signals of bone marrow and neural-crest-derived cells were obligatorily found in all ISR lesions and in the majority of primary lesions. As a key finding, the percentage of cells stained by CD34 and AC133 identifying endothelial progenitor cells in serial sections of ISR tissue was seven- to ninefold increased compared to primary lesions (CD34: 7.1 ± 2.5% vs. 0.6 ± 0.7%, \( P<0.001 \); AC133: 7.0 ± 3.4% vs. 1.0 ± 0.7%, \( P<0.001 \)). Frequency of dendritic cells (S100) was high with 9.8 ± 5.6% in ISR lesions, compared to 1.4 ± 1.1% \( (P<0.001) \) in primary plaques. Of note, these bone-marrow-derived cells were not confined to particular plaque regions or any predilection site. Likewise, expression of several markers of neural-crest-derived cells were again increased in ISR tissue (glial fibrillary acidic protein, GFAP: 7.9 ± 2.4% vs. 3.1 ± 1.0%; neuron-specific enolase, NSE: 4.4 ± 2.6% vs. 1.3 ± 1.6%; nerve growth factor receptor, NGFR: 4.2 ± 2.5% vs. 1.1 ± 0.7%; each \( P<0.001 \)). These markers were found to be equally distributed in all ISR samples without any predilection site.

![Image](https://example.com/image.jpg)

Fig. 3. (a) ISR tissue stained with unspecific mouse IgG1 as negative control to exclude nonspecific immunostaining. (b) Double staining for CD34 (yellow signal; white arrowheads) and AC133 (blue signal; cytoplasmic not nuclear signals indicated by white arrows). Note that AC133 is observed in a subset of CD34 positive cells and that all cells labelled by AC133 consistently express CD34. (c) Photomicrograph of double immunostaining for CD34 (blue signal, white arrow) and S100 (yellow signal, black arrow) demonstrating CD34'S100' neointimal cells. (d)–(f) illustrate magnified lesional details (boxes); bar = 50 μm.
consequence, about 5–15% of intimal cells did not stain with the specific cell type markers used, suggesting additional yet unidentified cell types and origins, respectively, in human ISR.

Importantly, double immunostaining did not demonstrate α-SM actin positive cells exhibiting CD34, AC133, S100, GFAP or NSE coexpression, respectively. With respect to EPC immunolabelling, AC133 could be detected in a subset of CD34 positive cells, whereas all cells stained by AC133 also expressed the CD34 antigen (Fig. 3b). Quantitatively, both EPC marker proteins revealed a significant correlation ($r = 0.57$, $P < 0.01$). There were only a few cells found in ISR atherectomy probes that showed coexpression of CD34 and S100 among several cells stained positive for either CD34 or S100, as illustrated in Fig. 3c–f. Likewise, if cells were positive of either EPC, DC or NCC markers, double immunostaining demonstrated coexpression in some intimal cells.

4. Discussion

The present report studies the presence of bone-marrow- and neural-crest-derived cells in atherectomy probes retrieved from ISR versus primary lesions from both the coronary and the peripheral vascular bed. A major finding of human ISR in coronary and peripheral vessels is its hypercellularity that significantly differs from de novo lesions and that is consistent with previous work demonstrating hypercellularity [5,17,19]. Traditionally, intimal hypercellularity and development of ISR has been attributed to migration, proliferation and mitigated apoptosis of smooth muscle cells (SMCs) [17,19]. Likewise, in the present study, ISR tissue was composed predominantly (67%) of α-SM actin positive cells.

As the central finding of the present study, signals for bone-marrow-derived endothelial progenitor cells (EPCs) and dendritic cells (DCs) as well as of neural-crest-derived cells (NCCs) were found in all atherectomy probes with ISR origin (Fig. 1). Furthermore, specific staining was increased two- to ninefold in ISR tissue compared to de novo plaques (Fig. 2), suggesting the recruitment of primarily extravascular cells to contribute to neointima formation after stenting. Indeed, a recent study on neointimal lesions 4 weeks after severe arterial traumatization in mice revealed up to 56% of cells with bone-marrow origin that were equally distributed in the intima [8]. In our present study, we extended these observations by demonstrating an average frequency of EPCs positive for CD34 and the more immature marker protein AC133 of 7% in specimens retrieved from patients with clinical ISR. Our findings of both colocalization and significant correlation of CD34 and AC133 (Fig. 3b) indicate the presence of common endothelial progenitor cells, as supported by recent work [9,20]. However, it remains open whether EPCs actually promote the endothelialization of injured target lesions and thereby contribute to vascular healing, as favored recently [21], and/or contribute to neointima formation and ISR volume expansion.

Our study demonstrates, for the first time, the presence of S100 positive dendritic cells (DCs) in the context with ISR neointima formation. Interestingly, DCs may also differentiate from CD34+ hematopoietic progenitors [22] which is in agreement with our finding of a few CD34+S100+ neointimal cells (Fig. 3c–f), favoring common progenitor cells that differentiate in endothelial and dendritic precursor cells. Recently, two reports detected DCs in carotid plaques [11] and diseased aorta [23] of rats with diet-induced hypercholesterolaemia, supporting a role of DCs in processing immune information and inflammation in atherogenesis. Furthermore, within normal intima from young adults, vascular-associated DCs accumulated most densely in those regions that were known to be predisposed for subsequent lesion formation [24]. Another remarkable finding was that the expression of neural-crest markers (GFAP, NSE, NGFR) was more extensive in ISR tissue than in primary lesions (Fig. 2). Interestingly, neural-crest-derived cells (NCCs) are known to be the source of SMCs specifically in large thoracic blood vessels [14,25–27]. Our data suggest that NCCs may also give rise to neointimal cells in the coronary and peripheral vascular bed.

In the present report on clinical ISR, we demonstrate that the predominant proportion of neointimal cells (67%) bear the marker α-SM actin, whereas up to 10% of the residual cells reveal distinct signals for bone-marrow- or neural-crest-derived cells (Figs. 1 and 2). Christen et al. [28] showed increasing percentages of mature smoothelin+ SMCs of 11% at day 7 and of 94% at day 30 in injured porcine coronary arteries. Therefore, one may speculate that progenitor cells with time may transdifferentiate into SMCs and/or other more differentiated forms of bone-marrow- and neural-crest-derived cells (Figs. 1 and 2). Christen et al. [28] showed increasing percentages of mature smoothelin+ SMCs of 11% at day 7 and of 94% at day 30 in injured porcine coronary arteries. Therefore, one may speculate that progenitor cells with time may transdifferentiate into SMCs and/or other more differentiated forms of bone-marrow- and neural-crest-derived cells within the intimal soil [29–31]. Indeed, Sata et al. [20] observed that bone-marrow-derived cells were negative for markers of SMCs and endothelial cells, when they attached to the luminal site of the artery 1 week after the mechanical injury in mice. Others proved CD34 to be absent from adult human SMCs [29]. Likewise, we did not detect α-SM actin positive cells that showed coexpression of CD34, AC133 and/or S100, GFAP, NSE.

Of course, observations on human tissue obtained by DCA are limited to the specific time point of intervention and therefore cannot assess the early response after stenting that is nicely studied in longitudinal animal experiments. In addition, the number and volume of atherectomy probes is generally small compared to surgically excised plaque tissue. However, the circumscript therapeutic removal of the limiting target lesion as accomplished by DCA allows to examine plaque regions of interest as this is known for biopsies excised from diseased substrate. Notably, our ex vivo data document that all findings on human ISR tissue...
originating from coronary or peripheral lesions were independent of vascular bed.

In summary, the present study suggests that bone-marrow- and neural-crest-derived cells, including dendritic cells, contribute to neointima formation in ISR. With respect to potential clinical implications, our findings favor new therapy modalities to target mobilization, homing and differentiation of cells with bone-marrow and neural-crest origin. Transient myelosuppression and inhibition of chemokines that recruit cells of hematopoietic lineage, while having no effect on differentiated medial SMCs, recently showed to effectively prevent neointimal hyperplasia [32,33]. Clinical trials are mandatory to prove these novel attractive concepts for prevention of human ISR.

Acknowledgements

The authors gratefully acknowledge the expert technical assistance of Nicole Kuhn. This work was supported in part by the Bundesministerium für Bildung und Forschung (project 01KV9915) and the Deutsche Forschungsgemeinschaft (DFG Ba 1076/2-2).

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