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Correlation between Proliferating Cell Nuclear Antigen Expression and Phenotypic Change in Smooth Muscle Cells during Development of Coronary Arteriosclerosis in the Transplanted Heart

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Abstract It is well known that coronary arteriosclerosis after heart transplantation is concentric and rich with smooth muscle cells (SMCs) ; however, the role played by rejection in the intimal thickening caused by SMCs in coronary arteriosclerosis remains unclear. In this study, we examined the process of intimal hyperplasia caused by SMCs and the relationship between the differentiation state of SMCs and the local inflammation caused by rejection. Lewis rat hearts were heterotopically transplanted into F 344 rats (allo-transplantation group) or other Lewis rats (iso-transplantation group). Cyclosporine A, 5 mg/kg/day, was injected intramuscularly for 20 days after transplantation in both groups. The transplanted hearts were examined immunohistochemically using several monoclonal antibodies, HHF-35, CGA 7, vimentin, and PCNA. To evaluate the grade of local immunological response caused by rejection, the anti-proliferating cell nuclear antigen (PCNA) antibody was used. In the allo-transplantation group, SMCs in the media began to undergo a phenotypic change toward a poorly differentiated state 30 days after transplantation. Intimal hyperplasia was observed 60 days after transplantation ; the thickened intima being composed mainly of de-differentiated SMCs with abundant PCNA-positive cells. The state of differentiation of SMCs in the thickened intima 90 days after transplantation varied from a de-differentiated to a highly differentiated state. These changes were well correlated with the expression of PCNA. The expression of PCNA was well correlated with the differentiation state of SMCs. Thus, the local inflammation caused by rejection may play an important role in the initiation of phenotypic change in SMCs.

Key words :Heart transplantation, coronary artery, arteriosclerosis, rejection, smooth muscle cell.

Introduction

Although graft survival following heart transplantation has been improved remarkably with the development of immunosuppressive therapy, the development of arteriosclerosis of the coronary arteries in the transplanted heart is still one of the serious complications of heart transplantation[1]. The findings of several experimental models of arteriosclerosis of the coronary and renal arteries have suggested that arteriosclerosis in transplanted hearts and kidneys is related to an immunological response caused by chronic rejection[2-6]. Previous reports by our group and others have shown that SMCs contribute to the intimal thickening that occurs in the transplanted heart[7,8]. Moreover, SMCs were

found to migrate through the injured internal elastic lamina to the intima forming its hyperplasia[8,9].

The phenotypic changes of SMCs were observed in human renal allografts[10,11], but have never been reported in the coronary arteries of transplanted hearts. Thus, in the present study, we examined the phenotypic change of SMCs in the coronary arteries of transplanted hearts using an immunohistochemical analysis of the same monoclonal antibodies as those employed in our previous studies on human renal allografts[10,11].

Several immunological factors have been reported to cause the intimal thickening in transplanted coronary and renal arteries[4-6]. Persistent local inflammation due to rejection might be a trigger leading to the development of intimal hyperplasia. It has been reported that PCNA plays a role as an immunoreactive nuclear antigen in the early stages of the inflammation[12-13] and that the up-regulated expression of PCNA precedes graft rejection[14]. Therefore, in the present study, the expression of PCNA was evaluated as an indicator of local inflammation after heart transplantation, and compared with the changes that occur in the differentiation state of SMCs.

Materials and Methods

Animals

Inbred male F 344 (RT-1 ^{lvl}) and Lewis (RT-1^{l}) rats were obtained from Seiwa Experimental Animals Co., Ltd (Japan). Rats aged between 8 and 12 weeks old were used for all experiments.

Heart Transplantation

Lewis hearts were transplanted into F 344 rats (allo-transplantation group) or into other Lewis rats (iso-transplantation group). This experiment was approved by the Committee of the Ethics on Animal Experiments of the Yamaguchi University School of Medicine and carried out under the Guidelines for Animal Experiments of Yamaguchi University School of Medicine and the law (No. 105) and notification (No.6) of the government.

Heterotopical heart transplantation into the abdominal cavity was performed using the modified Ono and Lindsey technique[15] under ether anesthesia. The transplantation technique utilized an end-to-side anastomosis of the donor's pulmonary artery to the recipient's inferior vena cava and the donor's ascending aorta to the recipient's abdominal aorta. Graft survival was monitored by daily palpation of the graft through the abdominal wall. Cyclosporine A, 5 mg/kg/day, was injected intramuscularly for 20 days after transplantation in both the iso- and the allo-transplantation groups. Moreover, sham-operated rats were used in the control group.

Preliminary experiments demonstrated that five of six grafts survived for more than 100 days in the allo-transplantation group of rats (n=5) which had received cyclosporine A. In the iso-transplantation group (n=5), all the grafted hearts lasted longer than 100 days.

Histological Analysis

The transplanted rats were sacrificed 30 (n=5), 60 (n=5) and 90 days (n=5) after transplantation in the allo-transplantation group and 90 days after transplantation in the iso-transplantation group (n=5). Transplanted hearts were removed and divided into four transverse slices, all of which were fixed in methanol-Carnoy's fixative. Tissue blocks were embedded in paraffin, and 20 serial sections from each block were cut at 5 μm thickness. Every first and second section was stained with hematoxyline-eosin and Weigert's elastic van Giesons stain, respectively, while the other sections were used for immunohistochemical staining.

Immunohistochemistry

To identify the SMCs and evaluate their differentiation state, three antibodies were used, namely : the anti-muscle actin monoclonal antibody HHF-35 (Enzo Diagnostic, Inc., New York, NY, USA) ; the anti-SMC actin monoclonal antibody CGA-7 (Enzo Diagnostic, Inc., New York, NY, USA) ; and the anti-vimentin monoclonal antibody

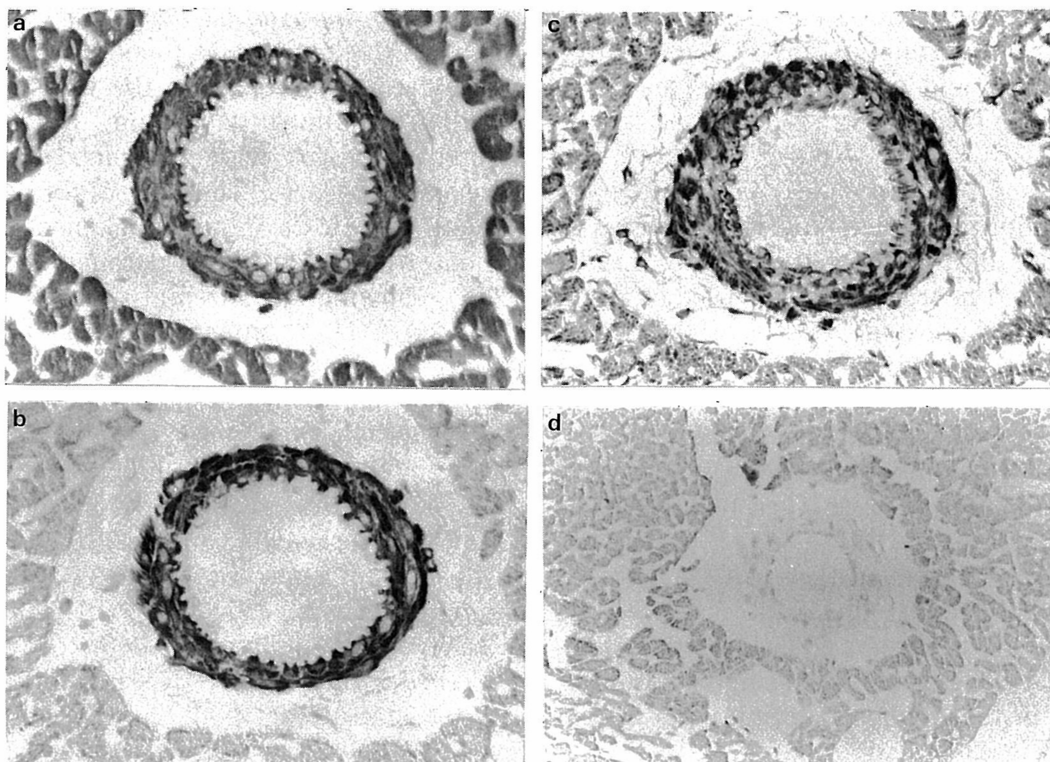

Fig 1. Microphotographs of the coronary artery in the control group
The slice sections were stained with the antibodies against HHF-35 (a), CGA-7 (b), VIM (c), and PCNA (d). SMCs of coronary artery were HHF-35^+ (a)/CGA-7^+ (b)/vimentin$^+$ (c)/PCNA$^-$ (d). Cardiac tissue was also PCNA- (d). (Original magnification × 150, × 90 (d)).

(DAKOPATTS, Inc., Kyoto, JAPAN). The antibody HHF-35 reacts with specific α-and γ-actin isotypes common to all muscle cells including SMCs ; the antibody CGA-7 recognizes specific α-and γ-actin isotypes specific to SMCs ; and the anti-vimentin antibody was used to identify mesenchymal cells. Moreover, the monoclonal antibody against proliferating cell nuclear antigen (PCNA, Dako A/S, Glostrup, Denmark), which is expressed in the late G 1 (presynthetic), S(DNA synthetic), and G 2 (premitotic) phases of the cell cycle, was used to detect proliferating cells. The expression of PCNA was estimated as an indicator of local inflammation. The specificity of these antibodies has been previously reported[16-20]. Highly differentiated SMCs are HHF-35^+/CGA-7^+/vimentin$^+$/PCNA$^-$, poorly differentiated SMCs are HHF-35^+/CGA-7^-/vimentin$^+$/PCNA$^-$, and de-differentiated SMCs are HHF-35^-/CGA-7^-/vimentin$^+$/PCNA$^+$[21-23]. The labeled streptavidin-biotin complex system with nickel chloride color modification was employed in all instances[24]. Sections were counterstained with methyl green.

Results

Histology of the control Group

The hearts of sham-operated rats were revealed that SMCs of almost all the coronary arteries were HHF-35^+/CGA 7^+/vimentin$^+$/PCNA$^-$(Fig. 1-a, b, c, d). Moreovere, cardiac tissue of sham-operated rats was PCNA$^-$(Fig. 1-d).

Histology of the Allo 30-day Group

Intimal hyperplasia in the coronary arteries was not detected in the transplanted hearts of the allo 30-day group (Fig. 2). The majority of medial SMCs showed a poorly differentiated phenotype of HHF-35^+/CGA-7^- (Fig.

2-a, b), as observed in all transplanted hearts in allo 30-day group. A few PCNA-positive cells were found occasionally at the inner layer of the media (Fig. 2-c).

Histology of the Allo 60-day Group

Severe intimal thickening was detected in almost all of the coronary arteries in the transplanted hearts of the allo 60-day group (Fig. 3). Almost all SMCs of the media showed a poorly differentiated phenotype of HHF-35^+/CGA-7^-/vimentin$^+$/PCNA$^-$ in allo 60-day all transplanted hearts (Fig. 3-b, c, d, e). SMCs in the thickened intima showed a de-differentiated phenotype of HHF-35^-/CGA-7^-/vimentin$^+$/PCNA$^+$ in all transplanted hearts in allo 60-day group (Fig. 3-b, c, d, e). The number of PCNA-positive cells increased in the cardiac tissue, with an abundance of PCNA-positive cells in the coronary arteries in the thickened intima, but not in media (Fig. 3-e).

Histology of the Allo 90-day Group

Intimal thickening was found in almost all of the coronary arteries in the transplanted hearts of the allo 90-day group. Four to six coronary arteries were examined in each transplanted heart. The state of differentiation of the SMCs in the thickened intima varied, with 18 out of 25 coronary arteries (72%) showing de-differentiated SMCs of HHF-35^-/CGA-7^-/vimentin$^+$/PCNA$^+$ (Fig. 4-a, b, c, d), and other coronary arteries showing highly differentiated SMCs of HHF 35^+/CGA 7^+/vimentin$^+$/PCNA$^-$ (Fig. 5-a, b, c, d). On the other hand, phenotypic change of the medial SMCs of almost all the coronary arteries were returned to a highly differentiated state of HHF-35^+/CGA 7^+/vimentin$^+$/PCNA$^-$ (Fig. 4, 5). Abundant PCNA-positive cells were detected in the thickened intima with SMCs in a de-differentiated state (Fig. 4-d) ; however, only a few PCNA-positive cells were found in the thickened intima with SMCs in a highly

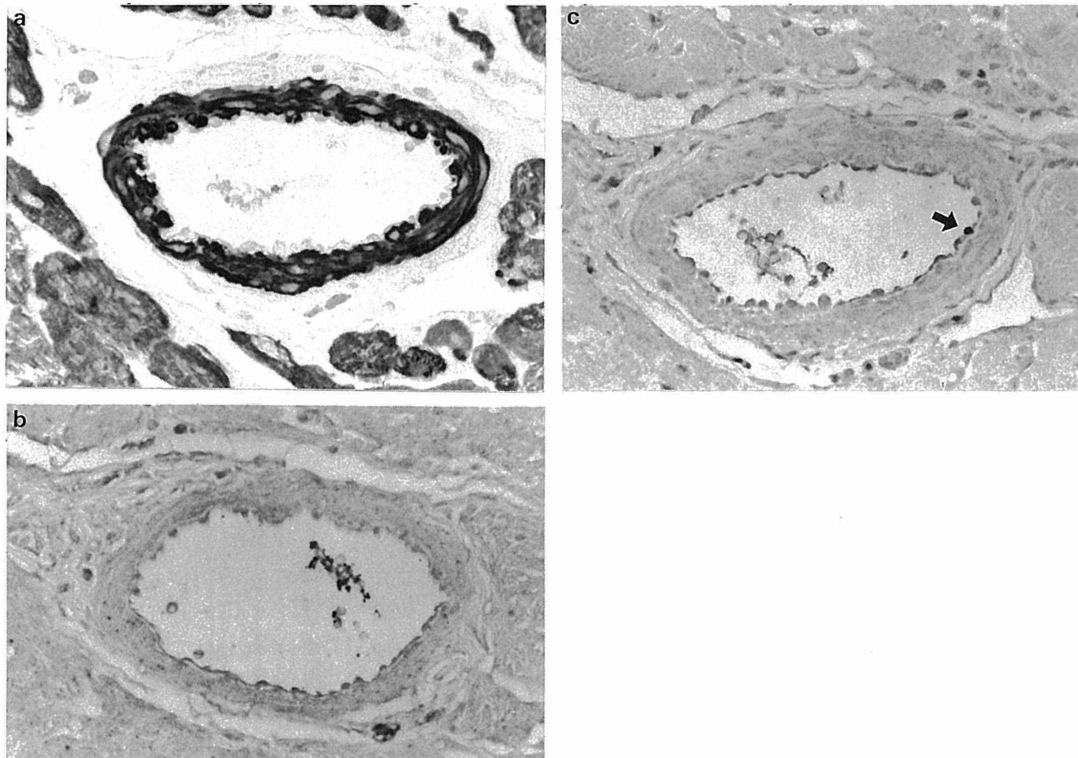

Fig 2. Microphotographs of the coronary artery in the allo 30-day group
The slice sections were stained with the antibodies against HHF-35 (a), CGA-7 (b), and PCNA (c). SMCs in the media showed a HHF-35^+ (a)/CGA-7^- (b). A few PCNA-positive cells were found at the inner layer of the media (c) (arrow). (Original magnification $\times$ 125).

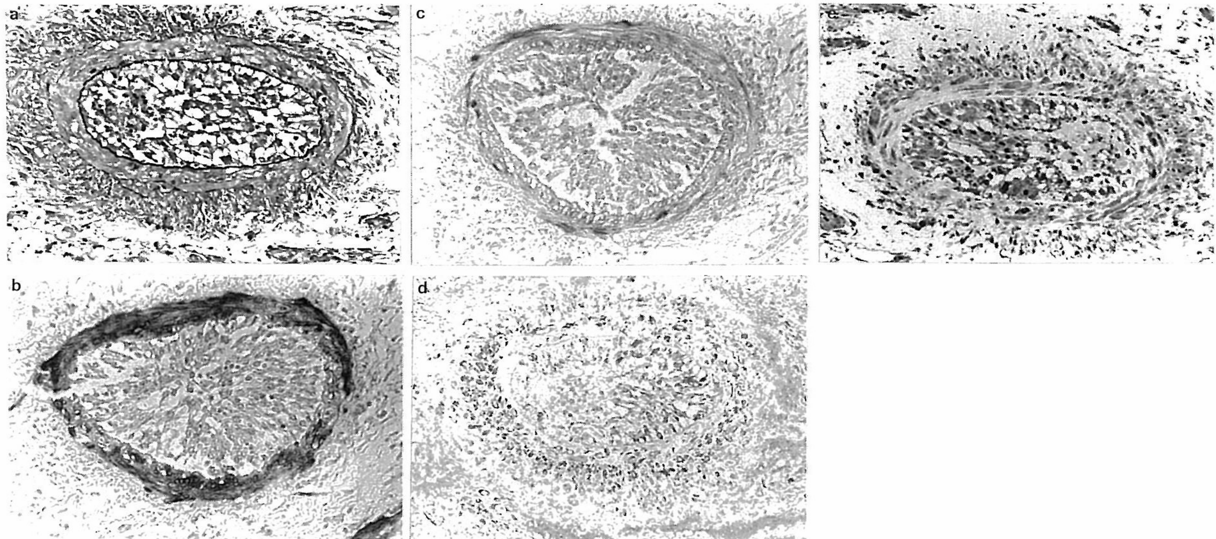

Fig 3. Microphotographs of the coronary artery in the allo 60-day group
The slice sections were stained with the antibodies against HHF-35 (b), CGA-7 (c), VIM (d), and PCNA (e). Severe thickened intima was detected in the coronary artery (a) (Weigert's elastic van Giesons stain). Phenotype of the medial SMCs were HHF-35^+ (b)/CGA-7^- (c)/VIM^+ (d)/$PCNA^-$ (e). SMCs in the thickened intima were HHF-35^- (b)/CGA-7^- (c)/VIM^+ (d)/$PCNA^+$ (e). (Original magnification × 150).

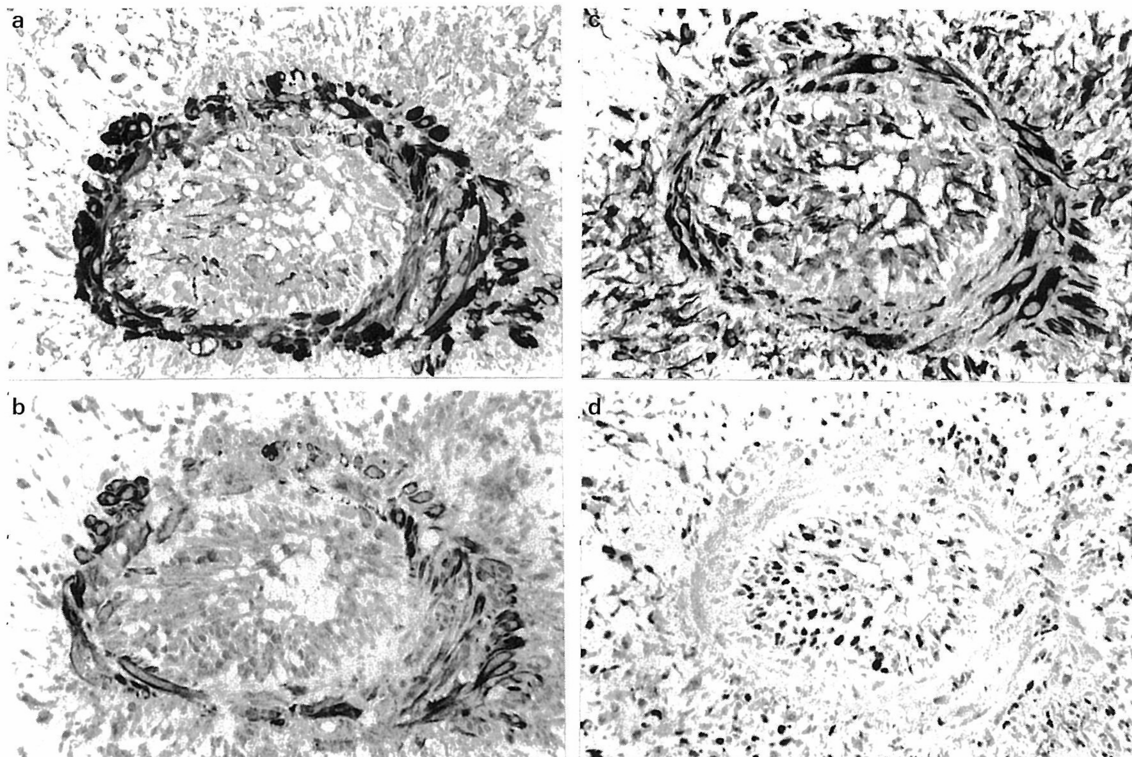

Fig 4. Microphotographs of the coronary artery with de-differentiated SMCs in the allo 90-day group
The slice sections were stained with the antibodies against HHF-35 (a), CGA-7 (b), vimentin (c), and PCNA (d). SMCs in the severe thickened intima were HHF-35^- (a)/CGA-7^- (b)/$vimentin^+$ (c)/$PCNA^+$ (d) in the coronary artery. SMCs of the media were HHF-35^+ (a)/CGA-7^+ (b)/$vimentin^+$ (c)/$PCNA^-$ (d). (Original magnification x 150).

differentiated state (Fig. 5-d).

Histology of the Iso 90-day Group

Intimal hyperplasia was not detected in the coronary arteries of the transplanted hearts in the iso 90-day group (Fig. 6-a). PCNA-positive cells were not observed in the coronary arteries (Fig. 6-b).

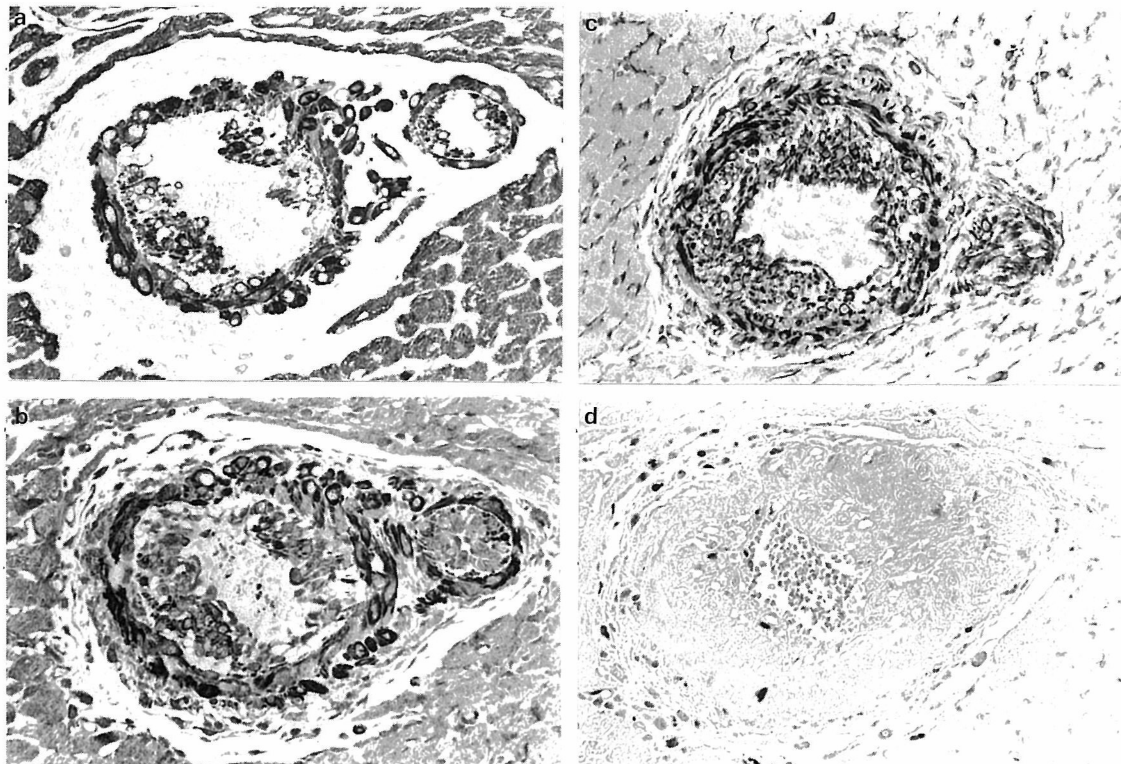

Fig 5. Microphotographs of the coronary artery with differentiated SMCs in the allo 90-day group
The slice sections were stained with the antibodies against HHF-35 (a), CGA-7 (b), vimentin (c) and PCNA (d). The SMCs in the thickened intima and the media showed HHF-35^{+} (a)/CGA-7^{+} (b)/vimentin^{+} (c)/PCNA^{-} (d) in the coronary artery. (Original magnification × 150).

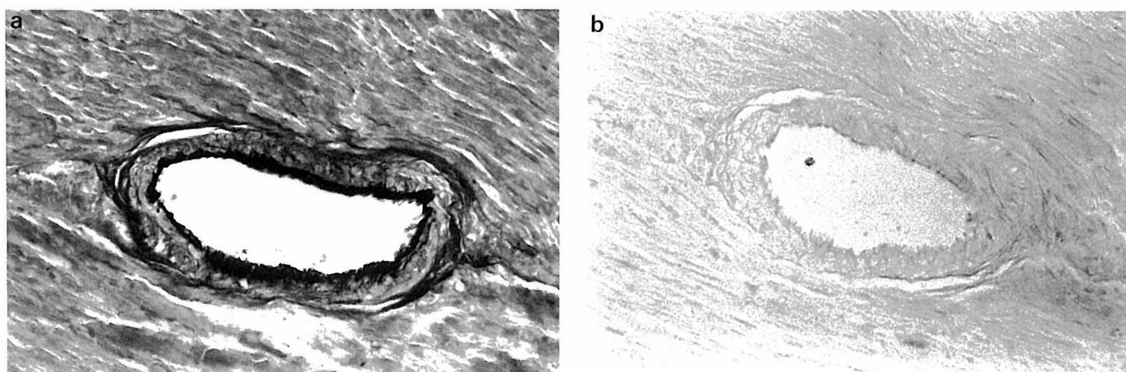

Fig 6. Microphotographs of the coronary artery in the iso 90-day group
The slice section was stained with the antibody against PCNA (b). Thickened intima was not detected in the coronary artery (a) (Weigert's elastic van Giesons stain). PCNA-positive cells were not detected in the specimen (b). (Original magnification × 150).

Discussion

Arteriosclerosis of the coronary arteries is one of the major clinical complications of heart transplantation[1]. Chronic rejection is thought to play an important role in the pathogenesis of arteriosclerosis. Humoral responses involving immunoglobulin, complement, and antigen-antibody complex ; and cellular responses involving lymphocytes and macrophages, have been reported as contributing factors in the development of arteriosclerosis after transplantation[4-6]. Moreover, previous report by our group had shown that proliferating SMCs derive from the media through the disrupted internal elastic lamina to the intima[8]. In this study we examined the process of intimal thickening caused by SMC proliferation, and the rejection-related phenotypic change of SMCs. In addition, the extent of local inflammation was evaluated by the expression of PCNA. The technique used in the present study to distinguish the SMC phenotype has been described in previous reports[21-23]. PCNA is a DNA polymerase δ accessory protein[12] that is expressed within the nuclei of proliferating cells and is absent from cells not actively involved in DNA synthesis[25]. Because the expression of PCNA is up-regulated by preceding rejection[14], we used PCNA as an indicator of local inflammation caused by rejection.

The findings of the present study revealed that the phenotype of the SMCs in the media of the coronary arteries in the allo-transplantation group had already changed from a highly differentiated state to a poorly differentiated state by 30 days after transplantation. Interestingly, at this stage, poor differentiation of the SMCs in the media and a few -PCNA positive cells at the inner layer of the media were found. This strongly suggests that phenotypic change of the SMCs, which occurs in the coronary arteries of transplanted hearts, is closely related to the local inflammation caused by rejection.

By 60 days after transplantation, phenotypic change of the medial SMCs was a poorly differentiated state and neointimal tissues composed mainly of de-differentiated SMCs were found in almost all the coronary arteries. At this stage, the coronary arteries showed abundant PCNA-positive cells in the thickened intima.

By 90 days after transplantation, once re -differentiation of the SMCs had occurred in the thickened intima, PCNA-positive cells were rarely found. Because, SMCs in the thickened intima showed a de-differentiated phenotype in allo 60-day all transplanted hearts, but 28% of coronary arteries in allo 90-day group showed a highly differentiated phenotype. These observations strongly suggest that poor differentiation of medial SMCs in coronary arteries occurs during the early stage after heart transplantation, followed by the formation of neointima, and at later stages, by the gradual re-differentiation of neointimal SMCs to a mature phenotype. Phenotypic change of the medial SMCs of almost all the coronary arteries was re -differentiation type at this stage. Therefore, it was suggested that the medial SMCs returned to mature state. Furthermore, the findings of the present study indicate that the re-differentiation of neointimal SMCs appears to be related to cessation of the rejection response, associated with the disappearance of PCNA-positive cells within the neointima.

Our observations of the rat heart transplantation model described herein are relevant in that almost identical shifts in the cytoskeletal phenotype of SMCs during the development of neointima were demonstrated, using the same monoclonal antibodies as those used in the intrarenal arteries of human renal allografts[10,11].

Although the subtypes of the PCNA-positive cells were not analyzed, in this study, there were probably T cells and macrophages in the tissues of allograft. It has been reported that macrophages are able to stimulate SMCs to change their phenotype to a synthetic state[26,27]. It is likely that activated T cells and macrophages produce several cytokines and growth factors which may stimulate phenotypic change in SMCs.

In conclusion, this is the first report to suggest an important role of the rejection-induced local inflammation (as assessed by the PCNA expression) in the initiation of the phenotypic change of SMCs in transplanted hearts.

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