学 位 論 文

Pathological Analysis of Cell Differentiation in Cholesterin Granulomas experimentally induced in Mice

（実験的に誘発したマウスのコレステリン肉芽腫における細胞分化に関する病理学的検討）

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松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

Pathological Analysis of Cell Differentiation in Cholesterin Granulomas experimentally induced in Mice

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《要旨》

【諸言】コレステリン肉芽腫には多数のマクロファージと多核巨細胞が出現する。しかし，その細胞性格について，さらに，線維芽細胞や毛細血管内皮細胞がどこから供給されるのかなどの知見は乏しい。そこで，実験的にコレステリン肉芽腫を惹起させ，増殖する細胞種とその動態を明らかにすべく病理学的に検討した。

【材料・方法】ddY マウス（7 週齢）と GFP 骨髄移植マウスを用い皮下にコレステリン 10mg を埋入し，埋入 2 週間から最長 6 か月まで病理組織学的，免疫組織化学的，ならびに免疫蛍光二重染色により比較検討した。

【結果】病理組織学的には ddY マウスと GFP 骨髄移植マウスを用いたがその病理組織像について相違はなかった。埋入 2 週例では，肉芽組織がコレステリン結晶の塊の周囲から一部を置換していた。その中心部は大きな不規則な形状の空隙として観察された。これらの細胞は主に，マクロファージや多核巨細胞であった。大きな空隙に接する内部に毛細血管はほとんどなかった。大きな空隙に接する部分ではマクロファージや多核巨細胞の核の染色性は極めて悪かった。3 週例では，さらに中心部に行くに従いマクロファージや異物巨細胞の増殖があったが細胞核の消失により細胞形態を有せず，ただコレステリン結晶を分割するような構造とし観察された。3 か月例では，器質化していた。これらの部には，線維芽細胞と膠原線維と毛細血管の増生があり，コレステリンの分割化が進んでいた。最外層には線維芽細胞が膠原線維の走向に増殖しており，毛細血管もみられた。6 か月例では，中心にあった大きな不規則な形状のコレステリン結晶はほぼ線維芽細胞や膠原線維に置き換わっていた。最表層の被膜には，線維芽細胞と膠原線維と毛細血管が存在していた。免疫組織化学的検討では，CD68 について，コレステリン空隙の周囲に増殖した円形の核を持つ細胞や多核の巨細胞は明確に CD68 陽性を示した。埋入 2 週の増殖した肉芽組織の細胞はほぼすべて GFP 陽性であった。肉芽組織内には，極めて少量の線維芽細胞と膠原線維が介在しており，その線維芽細胞は GFP 陰性だった。しかし，6 か月経過すると，マクロファージと多核巨細胞の間には，多量の線維性組織が入り込み，これらを分割していた。その膠原線維と線維芽細胞間と毛細血管が介在していた。この大部分は GFP 陰性を示した。しかし，一部の紡錘形の核を持つ線維芽細胞は GFP 陽性を示した。次に，CD31 陽性細胞はほぼ肉芽組織の周辺部に限局していた。最外層の線維性組織内には内層と比べ多くの CD31 陽性が認められた。免疫蛍光二重染色による検討では，GFP-CD68 の組み合わせについて，CD68 陽性のマクロファージと異物巨細胞は GFP 陽性を明確に示した。紡錘形細胞の一部には GFP のみ陽性の細胞も存在していた。GFP-CD31 の組み合わせでは，一部の血管内皮細胞は GFP と CD31 の二重陽性を示した。

【考察】病理組織学的に埋入 2 週間例では，コレステリンの塊の残存による大きな空隙に接する部分ではマクロファージや多核巨細胞の核の染色性は極めて悪かった。これは，毛細血管がほとんど進入していないため細胞が壊死しているものと考えられた。3 か月例では肉芽組織に置換していた。コレステリン空隙の周囲に増殖した円形の核を持つ細胞や多核の巨細胞は明確に CD68 陽性を示した。興味深いのは，CD31 陽性の血管内皮細胞の GFP 陽性を呈するものが確認されたことである。今まで，血管内皮細胞が傷害刺激等により骨髄から移動してきた細胞から分化することに関して，若干の報告はあるが，これを明確に示したものはない。GFP-CD31 の蛍光二重染色による検討では，形態学的に明瞭な血管において，両陽性の血管内皮細胞が認められ，移植骨髄細胞由来である事が判った。以上の結果，毛細血管内皮細胞も骨髄間葉細胞由来であることが明らかになった。今回の実験系では 6 か月と言う長期にわたって肉芽組織形成が継続的になされていたためであろう。

Pathological Analysis of Cell Differentiation in Cholesterin Granulomas experimentally induced in Mice

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Abstract

Cell differentiation in cholesterin granulomas was investigated using ddY mice and GFP bone marrow transplanted mice. Cholesterin was embedded in mice subcutaneously and histopathological examination was carried out in a period of 6 months. Results showed that at 2 weeks, cholesterin was replaced partly by granulation tissues. The majority of cells in the granulation tissues were macrophages and foreign body giant cells and the center consists of small amount of fibroblasts, collagen fibers and capillaries. At 3 months, more granulation tissue was observed compared to 2 weeks. Similar cells were observed, however, there were more fibroblasts, collagen bundles and capillaries present compared to 2 weeks. At 6 months, the cholesterin was mostly substituted by fibrous tissues consisting mainly of fibroblasts and collagen fibers with some macrophages and foreign body giant cells. Specifically, the outer part of the tissue consists of fibroblasts, collagen bundles and capillaries and the inner portion is filled with collagen bundles. Immunohistochemistry revealed that macrophages and foreign body giant cells were positive to GFP and CD68 although the fibroblasts and capillaries in the outer portion of cholesterin granulomas were GFP negative. Some spindle shape fibroblasts were also GFP positive. Immunofluorescent double staining revealed that cells lining the blood vessels were both positive to GFP and CD31 indicating that those were endothelial cells and were actually derived from the transplanted bone marrow cells. The results suggest that macrophages, foreign body giant cells as well as fibroblasts and capillary endothelial cells are bone marrow derived mesenchymal cells.

Introduction

Granulation tissue formation is a part of secondary wound healing and tissue repair in vivo. Many studies have shown the formation of foreign body granulomas in vivo. For instance, charcoal (1), silicone (2, 3) and other foreign matter (4-6) induced the formation of foreign body granulomas. Cholesterin granuloma is a term given to foreign body granuloma present in various lesions caused by cholesterin (3, 7). The most common cells found in cholesterin granulomas are macrophages and foreign body giant cells (FBGC). However, the source of these cells as well as other cellular components such as fibroblasts and capillary endothelial cells has not been elucidated. Cholesterin is generally produced in the body. Therefore, cholesterin was experimentally induced and the tissue reaction was studied histopathologically with emphasis on cell migration and differentiation.

Materials and Methods

The animals used in this experiment were 7-week ddY mice purchased from Japan SLC, Inc (Hamamatsu, Japan), 7-week old, female, C57BL/6 GFP bone marrow transplanted mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) and 7-week old, female, C57BL/6T g (CAG-EGFP) GFP transgenic mice (Shimizu Laboratory Supplies, Kyoto, Japan). Bone marrow derived cells (BMDCs) were harvested by sacrificing GFP transgenic mice under ether anesthesia. Briefly, femurs were excised followed by soft tissue removal and harvest of BMDCs. The cells were washed with RPMI 1640 and then the medium was replaced with HBBS. After X-ray irradiation of GFP mouse with 10 Gray, the cells ($1x10^7$) were implanted in 7-week old female cognates from the tail vein. The engrafted mice were used 4 weeks after transplantation (8, 9).

The mice were placed under Isoflurane inhalation anesthesia and 10 mg of cholesterin was implanted subcutaneously by making an incision on the back of the mouse and then closed with sutures. After 2 weeks, 3 weeks, 3 months and 6 months, the embedded tissue was removed en bloc and fixed in 10 % neutral buffered formalin. The examined animals are shown in Table 1.

Histopathological examination

The fixed samples were dehydrated in series of alcohol, embedded in paraffin and sectioned into 5μm. Then after, specimens were stained with hematoxylin and eosin and examined under a microscope.

Immunohistochemistry

After deparaffinization, specimens were immersed in citric acid buffer solution with pH 6.0 (LSI Medience Co., Tokyo, Japan) and placed in autoclave at 121°C for 10 min. Blocking was carried out using serum-free protein block (Dako Japan Co., Ltd, Tokyo, Japan) at room temperature for 30 min. Slides were incubated with primary antibodies; anti-CD68 for detection of macrophages (1:100; ab125047, Abcam, Cambridge, UK), anti-CD31 for detection of endothelial cells (1:100; ab28364, Abcam, Cambridge, UK), anti-GFP Chlp Grade (1:2000; ab290, Abcam, Cambridge, UK) at 4°C overnight. This was followed by secondary antibody using anti-rabbit Ig for 30 min. Finally the slides were washed with PBS and developed with DAB.

Immunofluorescent double staining

After deparaffinization, slides were pre-treated in citrate buffer in microwave for 1 min and blocked with 10 % donkey normal serum for 30 min at room temperature. Primary antibodies anti GFP, anti-CD31, anti-CD68 were each diluted at 1:100 with Can Get Signal (Toyobo Co., Ltd, Osaka, Japan) and allowed to react overnight at 4 °C.

For secondary antibody, Alexa Fluor 568 Labeled Donkey Ant-goat Ig G antibodies (Life Technologies, Palo Alto, CA, USA) and Alexa Fluor 488 Labeled Donkey Anti-rat IgG Antibodies (Life Technologies, Palo Alto, CA, USA) and Can Get Signal (Toyobo Co., Ltd, Osaka, Japan) were diluted at 1:200 and allowed to react for

60 min at room temperature. Then after, the nuclei were stained with 1 mg/ml of DAPI for 3 min. Slides were then washed with TBS and mounted using Fluorescent Mounting Medium (Dako Japan Co., Ltd, Tokyo, Japan).

The study conformed to the experimental guidelines in animal experiment of Matsumoto Dental University approved by the Animal Laboratory Examination Committees of the University.

Results

Histopathological evaluation (Fig. 1)

There was no difference between ddY mice and GFP bone marrow transplanted mice histopathologically. At 2 weeks, the growing mass of granulation tissues surrounded the irregularly shaped cholesterin spaces (Fig. 1-a). The cholesterin spaces were actually the areas previously occupied by cholesterin crystals dissolved during tissue preparation. The growth of granulation tissue replaced some of the mass of cholesterin crystals. The granulation tissue primarily consists of macrophages and FBGCs. Within the granulation tissues, large and small blood vessels were observed. FBGCs have large vacuolated cytoplasm. Few fibroblasts and collagen bundles were observed in between macrophages and FBGCs. Some capillaries were also seen especially in the outer portion of granulation tissues where cell proliferation was evident. However, cell proliferation was not observed in the center of granulation tissue where large cholesterin spaces were still present. Moreover, nuclear staining of macrophages and FBGCs in contact with large vessels near residual cholesterin was extremely poor. The outer layer of granulation tissue has relatively thick fibrous tissue formation. There was proliferation of fibroblasts producing collagen fibers as well as capillaries.

At 3 weeks, the granulation tissues became bigger replacing more of the embedded cholesterin crystals. This resulted to a smaller irregular space in the center (Fig. 1-b). Cell morphology was not apparent due to the loss of nucleus in macrophages and FBGCs. The partition between the cell proliferation and remaining cholesterin spaces was still apparent. Many small and large blood vessels were spotted at the periphery of the granulation tissue. Moreover, capillaries were seen in between fibroblasts and collagen fiber in relatively thin peripheral layer of fibrous tissue.

At 3 weeks, the granulation tissue replaced clumps of embedded cholesterin crystals. Granulation tissue had grown and replaced the small dividing mass of cholesterin crystals. Macrophages and FBGCs surround the cholesterin spaces. Hyperplasia of fibroblasts, collagen bundles and capillaries separating the cholesterin spaces were noted (Fig. 1-c). The outer layer was also filled with fibroblasts, collagen bundles and capillaries.

At 6 months, granulation tissue has replaced the entire cholesterin crystals mainly composed of fibroblasts and collagen bundles. The periphery was replaced with finely organized fibrous tissues. Fibroblasts and collagen bundles also penetrated the center of the granulation tissues. The outer layer was composed of fibroblasts, collagen bundles and capillaries but mostly collagenbundles (Fig. 1-d).

Immunohistochemistry (Fig. 2)

Macrophages and FBGCs in the granulation tissue were positive to CD68 (Fig. 2-a). At 2 weeks, the granulation tissue was filled with GFP-positive cells. However, some of the fibroblasts in the granulation tissues were GFP negative. Relatively thick fibrous tissue was present in the outer layer of the granulation tissues.

At 6 months, a large amount of fibrous tissue separated the macrophages and FBGCs (Fig. 2-b). Fibroblasts and capillaries distributed in the collagen bundles were mostly GFP negative. The fibroblasts, collagen fibers and capillaries in the outer layer did not express GFP. However, fibroblasts having spindle shaped nuclei were GFP positive (Fig. 2-b). At 2 weeks, the granulation tissue was formed within the spaces left by cholesterin crystals. Most CD31 positive cells were localized at the periphery of the granulation tissues but also few cells in the center were positive to CD31 (Fig. 2-c). Capillaries that were present in the granulation tissue were positive to CD31 (Fig. 2-d).

Immunofluorescent double staining (Figs. 3, 4)

Immunofluorescent double staining revealed that macrophages and FBGCs were both GFP (green) and CD68 positive (red) (Fig. 3-a, b). Double staining confirmed that both green and red fluorescence was observed on relatively large and irregular macrophages and FBGCs (Fig. 3-c). However, the spindle shape cells were only positive to GFP.

Immunofluorescent double staining with GFP (green) and CD31 (red) showed GFP expression in the cytoplasm of endothelial cells in distinct blood vessels (Fig. 4-a), and also in the cross-linked-macrophages and FBGCs. Double staining with CD31 and DAPI (blue) showed red fluorescence on vascular endothelial cells indicating CD31 expression. Nuclei were stained blue for DAPI (Fig. 4-b).

A number of GFP-positive cells were spindle or polygonal in shape and some were lining the lumen of the blood vessels. CD31 expression was observed as red fluorescence in endothelial cells lining the blood vessel. Double staining showed that vascular endothelial cells were both positive to GFP and CD31 (Fig. 4-c).

Discussion

Macrophages and FBGCs mainly gather in reaction to a large external matter producing the so-called foreign body granuloma. Clinical reports showed that charcoal (1) and silicone (2, 3) are some of the foreign materials that can induce foreign body granuloma formation. Previous studies showed the formation of granulation tissue induced by dermal filler (5), subcutaneous injection of acetate in the treatment of prostate cancer (10) and bone wax (11). Moreover, Nakahori et al reported a case of lung cancer from silicone granuloma (3). Kim JS et al (7) and Lsaacson B (12) described the formation of granulation tissue induced by cholesterin. Cholesterin granuloma is a granulation tissue growth on cholesterin crystals. Macrophages mainly proliferate in the granuloma and then coalesce to form multinucleated giant cells having similar characteristics with FBGCs.

In this study, cholesterin was implanted in the subcutaneous tissue in mice to induce the formation of cholesterin granuloma. Histological examination was carried out to determine the type and source of cells. Biological evaluation of several

materials have been made (13, 14) following the experimental method of Shoumura et al in which the foreign material was embedded in the back subcutaneous tissue (15). This process conformed to ISO standards (16). The tissue surrounding the embedded cholesterin was examined histologically within the period of 6 months.

At 2 weeks, granulation tissue partly replaced the cholesterin crystals. Staining of the nucleus of macrophages and FBGCs in contact with large residual cholesterin crystals was extremely poor. The cells were mostly in the degenerative stage and undergoing necrosis. At 3 weeks, granulation tissue replaced a large amount of cholesterin crystals. At 3 months, granulation tissue replaced almost the entire mass of cholesterin crystals.

Macrophages and FBGCs with round nuclei seen around the lumen of the blood vessels were positive to CD68 verifying their cell type. At 2 weeks, some cells in the granulation tissue were positive to CD31 but other cells at the periphery only expressed CD31 at 6 months. The early proliferation of macrophage in the center signifies that there was no proliferation of capillaries inside the granulation tissue. This explains the tissue degeneration and necrosis histologically.

The bone marrow transplantation mouse model was used since it was assumed that the cells would originate from the bone marrow (17-19). Clinical application on the pluripotency of BMDCs had already begun. In fact cell transplantation in the treatment of myocardial and cerebral infarctions has been started with the objective that the cells would differentiate into what they are expected. In the oral region, trials concerning bone regeneration using cultured mesenchymal stem cells from human bone marrow have been conducted. This was for the purpose of regeneration of the jaw with thickness and strength required for oral implant placement. Bone marrow mesenchymal cells of patients were cultured, harvested and then grafted for the purpose that the cells would differentiate into osteoblasts for bone formation. However, studies on regenerative medicine using BMDCs have only been reported in the reconstruction of a very limited area in bone tissue. It is expected that other areas would be explored in the future. Therefore, studies on the migration and cell differentiation of BMDCs for oral tissue regeneration would contribute to the advancement in regenerative medicine. Using GFP transgenic mice, cells that constitute the tissue would express GFP if the transplanted BMDCs differentiate into any cell. Hence, tracking of differentiated cells is possible.

Tsuijigiwa et al.'s study on pluripotent BMDCs revealed the migration and differentiation of mesenchymal cells into odontogenic and periodontal tissue cells (20). Transplanted GFP-positive BMDCs migrated to the oral region in a short period of time as early as a month after implantation. The same research group showed GFP positive cells in mice periodontal tissue, dendritic cells, Langerhans cells, osteoclasts etc. However, there is no clear data of differentiation into vascular endothelial cells (21, 22).

This experiment showed that macrophages and FBGCs were derived from transplanted bone marrow mesenchymal cells shown by their GFP expression. Remarkably, GFP positive cells also expressed CD31 indicating that they are endothelial cells. Several studies mentioned that bone marrow derived cells migrate to the site of injury and can differentiate into vascular endothelial cells however this

has not been clearly shown (17, 18, 19, 23, 24). Another interesting idea is the chronic inflammation and angiogenesis mostly observed in tumors. The continuous angiogenesis seems not only due to local endothelial cell proliferation but also from the movement of mesenchymal cells from the bone marrow (25-27). The formation of granulation tissue over a long period of time was due to the presence of cholesterin. Co-expression of GFP and CD31 was observed in vascular endothelial cells. Fibroblasts and endothelial cells in the granulation tissue expressed GFP suggesting that the cells were derived from the bone marrow. This was further supported by double immunofluorescent staining showing co-expression of GFP and CD31 in vascular endothelial cells seen in distinct blood vessels. In summary, the study presented that macrophages and FBGCs as well as some of the fibroblasts and capillary endothelial cells were bone marrow derived mesenchymal cells.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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Table 1. Periods and Number of Experimental Animals (ddY and GFP)

ddY	Experimetal					Total
	Periods	2 weeks	3 weeks	3 months	6 months	
	Number	3	3	3	3	12
GFP	Experimental					
	Periods	2 weeks	3 weeks	3 months	6 months	
	Number	1	1	1	1	4

Figure legends:

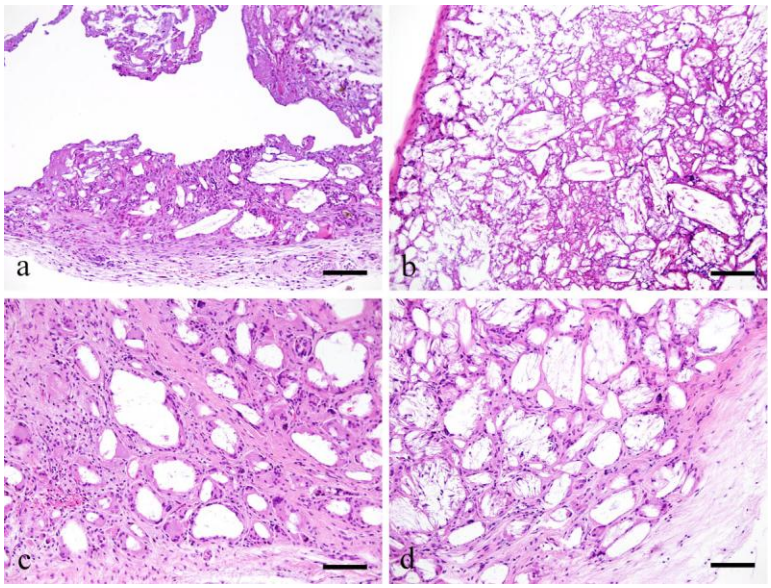

Figure 1. Histopathological views of ddY mice. a: 2 weeks; b: 3 weeks; c: 3 months, d: 6months; scale bars=100μm.

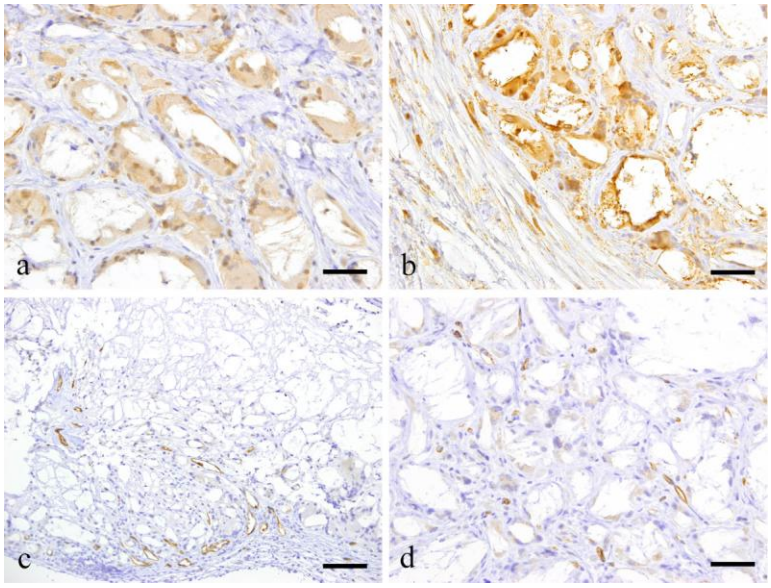

Figure 2. Immunohistochemical results. a: CD68 expression (3 weeks, scale bar=50μm); b: GFP expression (6 months, scale bar=50μm); c: CD31 expression (2 weeks, scale bar=100 μm); d: CD31 expression (6 months, scale bar=50μm).

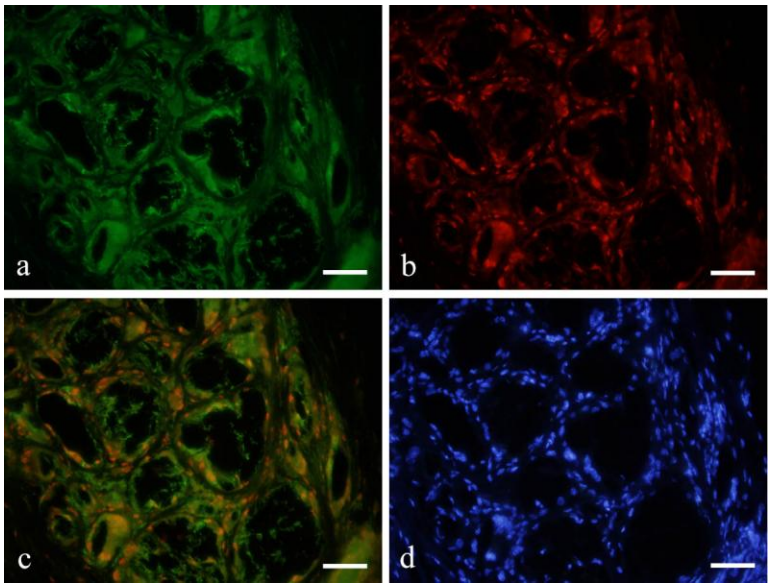

Figure 3. GFP, CD68 double immunofluorescent staining results. a: GFP; b: CD68; c: GFP+CD68; d: DAPI; 6months, scale bars=50μm.

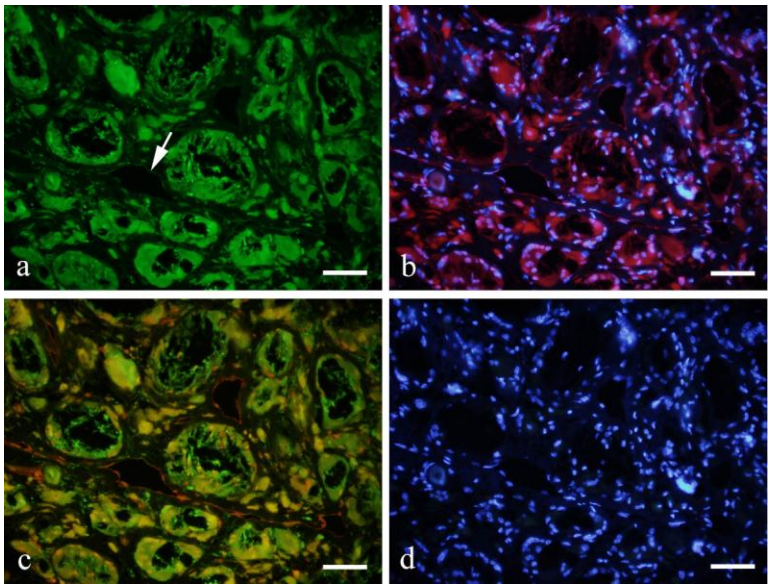

Figure 4. GFP, CD31 double immunoflluorescent staining results. a: GFP; b: CD31+DAPI; c: GFP+CD31; d: DAPI; 6months, scale bars=50μm.