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Original Article

Transient expression of cellular retinol-binding protein-1 during cardiac repair after myocardial infarction

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Retinoic acid (RA) is a vitamin A derivative that exerts pleiotropic biological effects. Intracellular transport and metabolism of RA are regulated by cellular retinol-binding proteins (CRBP). CRBP-1 is transiently expressed in granulation tissue fibroblasts during wound healing; however, its role in cardiac remodeling remains unknown. A rat myocardial infarction (MI) model was established by ligation of the left coronary artery, and hearts were obtained at 3, 6, 15, 30 and 45 days after operation. Heart sections were examined immunohistochemically using anti-vimentin, anti-α-smooth muscle actin (α-SMA), anti-matrix metalloproteinase (MMP)-2, anti-MMP-9 and anti-CRBP-1 antibodies. Infarction involved 48.8 ± 3.6% of the left ventricle and was followed by an important cardiac remodeling. Vimentin-positive fibroblastic cells including α-SMA-positive myofibroblasts expressed CRBP-1 at 3-, 6-, and 15-days after MI. Expression of CRBP-1 reached a maximum at 6-days after infarction. Thereafter, CRBP-1 expression was dramatically decreased, showing a similar tendency to MMP expression. Human heart specimens of individuals with a recent myocardial infarction demonstrated presence of CRBP-1-positive fibroblasts by immunohistochemistry. We have demonstrated that CRBP-1 is transiently expressed by fibroblasts during cardiac remodeling. Our results suggest that CRBP-1 plays a role in ventricular remodeling after MI allegedly through its RA binding activity.

Key words: cellular retinol-binding protein, fibrosis, myocardial infarction, ventricular remodeling

INTRODUCTION

Vitamin A plays a crucial role in the regulation of cell growth and differentiation.[1] Its active form, retinoic acid (RA), is involved in signal transduction pathways regulating embryonic development[2] and promotes cardiac differentiation.[3] It also modulates inflammation, immune reaction and metabolism of connective tissue through receptor-mediated changes in expression of genes, such as those of matrix metalloproteinases (MMP).[4] Retinol and RA are bound to cellular retinol-binding proteins (CRBP).[5] Intracellular transport and metabolism of vitamin A are regulated by CRBP[6] that are highly conserved in mammals[7,8] and belong to a family of cytosolic proteins binding small hydrophobic ligands.[9] CRBP-1 is expressed in liver, kidney, lung,[10] brain,[11] retinal pigment epithelium[12] and genital tract[13–15] of adults and it could play important roles in retinol metabolism steps, such as esterification,[16] oxidation,[17] and hydrolysis of retinyl esters into retinol.[18]

It is widely accepted that fibroblasts from different tissues may exhibit specific features, including varying degrees of differentiation and/or functional activity.[19–21] To classify different fibroblastic cells, vitamin A storage[22] and cytoskeletal protein expression[23] have proven useful. It is also known that during wound healing or in fibrocontractive diseases, fibroblasts modulate into myofibroblasts,[24,25] which are most numerous when wound contraction takes place and disappear through apoptosis after wound closure.[26] It has been reported that CRBP-1 is transiently expressed in fibroblasts cultured from different organs *in vitro* and granulation tissue during wound healing.[27]

Myocardial infarction (MI) and the consequent loss of cardiomyocytes is an important cause of chronic heart failure. Large infarcts induce cardiac remodeling including histological changes of both infarcted and residual myocardium.[28] The

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healing process after MI is essential for prognosis, and myocardial remodeling is closely related to the incidence of arrhythmias and sudden cardiac death.[29] During healing, inflammation is dominant at the initial step, followed by repair phenomena mainly organized by fibroblastic cells with increased matrix synthesis and tissue retraction. The balance between the synthesis and degradation of collagen during cardiac remodeling depends on its turnover, which is mainly regulated by myofibroblasts.[30] We have investigated chronologically the expression of CRBP-1 in a rat MI model and in human heart tissue of patients with MI, in order to understand better its role in connective tissue remodeling following myocardial injury.

MATERIALS AND METHODS

Animal model of myocardial infarction

Eight-week-old Wistar rats weighing 180–220 g were used for this study according to the protocol approved by the Institutional Animal Care and Use Committee in National Cerebral and Cardiovascular Center, Osaka, Japan. Myocardial infarction was induced as previously described.[31] Briefly, animals were anesthetized with pentobarbital sodium and artificial respiration was established with a small-animal respirator (Harvard model 680, Harvard Apparatus, South Natick, MA, USA). The heart was exposed through a left thoracotomy and left coronary artery ligation was performed with 6-0 prolene sutures. Sham-operated rats underwent the same surgical procedure without coronary artery ligation. Twenty animals per group were sacrificed at 3, 6, 15, 30 and 45 days after coronary ligation. Control animals were sacrificed at 6 days after sham operation.

Histological, immunohistochemical examination and Western blotting

For histological examination, 10 animals in each group were perfusion fixed with Histochoice (AMRESCO Inc., Solon, OH, USA) and the ventricles were cut into three transverse slices from apex to base. Tissues were embedded in paraffin and segments of hearts were cut into serial 3-μm sections. The sections were stained with hematoxylin-eosin (HE) and Masson's trichrome (MT) for evaluation of histology and infarct area. Left ventricle (LV) area and infarct area were measured with an Image Analyze System equipped with digital camera (Microscope System DP71, Olympus Co., Tokyo, Japan) and software (Winroof, Mitani Co., Fukui, Japan) using MT slides. Luminal dimension was measured by the major axis in each case. Cross-sectional area of LV was manually defined on acquired digital images at the mid level of the LV transverse section and corresponded to the area of interest. At the same level, blue pixels were automatically selected and corresponded to the infarct area. Results were calculated as infarct area/cross-sectional aera (%). For immunohistochemistry, the primary antibodies used were mouse monoclonal antibodies against α-smooth muscle actin (α-SMA, Dako, Glostrup, Denmark; working dilution 1:50), vimentin (Dako; working dilution 1:800), MMP-2, MMP-9 (Daiichi fine chemical, Toyama, Japan; working dilution 1:50, 1:5000, respectively) and polyclonal rabbit CRBP-1 antibody (produced by the Department of Pathology and Immunology, University of Geneva; working dilution 1:100). Heat-induced epitope retrieval was always performed. Using the Image Analysis System mentioned above, cross-sectional area of LV was manually defined and brown pixels, corresponding to the α-SMA-positive and CRBP-1-positive areas were automatically selected. Results were calculated as positive area for these antibodies/cross-sectional area (%). Ten hearts were dissected into right and left ventricles, followed by measurement of heart weight and LV luminal dimension. Tissue was immediately frozen in liquid nitrogen for protein extraction. Detection of CRBP-1 was performed by Western blot as previously described.[32]

Human heart tissue

Human heart tissue was obtained by autopsy and the study protocol was approved by the ethics committee in Hyogo College of Medicine, Nishinomiya, Hyogo, Japan. Histological sections of normal myocardium ($n = 5$), acute MI ($n = 5$), recent MI ($n = 3$), and old MI ($n = 5$) were stained with HE and MT. Acute, recent, and old MI were selected from the archives of autopsy cases, Department of Surgical Pathology, Hyogo College of Medicine, according to the following criteria: acute died within 2 weeks, recent died within 2 months and old died more than 6 months after onset of MI. Immunohistochemistry staining for vimentin, α-SMA and CRBP-1 was performed to identify the fibroblasts, myofibroblasts and CRBP-1-positive cells. Results are shown as mean ± SEM for continuous variables. For statistical analysis, differences between groups were evaluated using the chi-square or Fisher exact test (for categorical variables) and *t*-test (for continuous variables). Two-sided probability values less than 0.05 were considered significant.

RESULTS

Cardiac remodeling after MI in the rat model

The baseline characteristics of sham-operated and infarcted animals are given in Table 1. All animals in MI group devel-

Table 1 Baseline characteristics of rat myocardial infarction model

		Myocardial infarction (days after coronary ligation)				
	Control	3-day	6-day	15-day	30-day	45-day
Number of animals	10	10	10	10	10	10
Body weight (g)	205 ± 23	193 ± 13	185 ± 21	210 ± 29	260 ± 10	276 ± 16
Left ventricle						
Weight (mg)	413 ± 53	410 ± 63	444 ± 59	529 ± 68*	583 ± 57*	658 ± 66*
HW/BW (mg/g)	2.02 ± 0.06	2.13 ± 0.30	2.42 ± 0.32*	2.56 ± 0.43*	2.24 ± 0.19*	2.42 ± 0.23*
Right ventricle						
Weight (mg)	109 ± 14	143 ± 16*	132 ± 27*	165 ± 23*	177 ± 25*	216 ± 28*
HW/BW (mg/g)	0.53 ± 0.02	0.75 ± 0.11*	0.71 ± 0.13*	0.80 ± 0.14*	0.68 ± 0.10*	0.79 ± 0.10*
LV luminal dimension						
Length (mm)	3.05 ± 0.23	4.97 ± 0.63*	5.15 ± 0.55*	5.53 ± 0.36*	5.59 ± 0.58*	6.22 ± 0.46*
Length/BW (μm/g)	15.02 ± 1.2	25.75 ± 2.88*	28.20 ± 4.04*	26.92 ± 5.07*	21.51 ± 2.36*	22.52 ± 2.20*
Infarct area (%)	0	52.2 ± 4.2	52.1 ± 3.6	51.2 ± 4.0	48.5 ± 5.3	48.4 ± 3.6

Results are given as mean ± SEM *versus control, $P < 0.05$.
BW, body weight; HW, heart weight; LV, left ventricle.

oped infarction ranging from 43.3% to 58.1% (mean 48.4 ± 3.6%) and infarct sizes was similar for infarction groups at 3, 6, 15, 30, and 45 days. The RV weight (mg)/body weight (g) was significantly higher at all time points, at 3, 6, 15, 30, and 45 days after infarction (0.75 ± 0.11, 0.71 ± 0.13, 0.80 ± 0.14, 0.68 ± 0.10, 0.79 ± 0.10, respectively) compared to sham-operated animals (0.53 ± 0.02). The LV luminal dimension (mm)/body weight (g) at 3, 6, 15, 30, and 45 days after infarction (25.8 ± 2.9, 28.2 ± 4.0, 26.9 ± 5.1, 21.5 ± 2.4, 22.5 ± 2.2, respectively) was also markedly increased compared to that of sham-operated animals (15.0 ± 1.2). These data indicated a remarkable LV remodeling after MI (Fig. 1).

At 3 days after MI, histological examination showed coagulation necrosis of myocardium with massive neutrophils infiltration in the infarcted area. At 6 days, neutrophils and macrophages infiltration, and proliferation of fibroblastic cells were observed. At 15 days, macrophage dominant infiltration and enhanced fibroblast accumulation were identified. At 30 days, deposits of collagen fibers were apparent and at 45 days, most of the infarcted area was replaced by scar tissue.

Alpha-SMA, CRBP-1 and MMPs expression in rat and human MI

The heart of sham-operated animals (Fig. 2a) showed the presence of vimentin-positive interstitial fibroblast (Fig. 2b), whereas α-SMA (Fig. 2c) and CRBP-1 (Fig. 2d) were absent in fibroblastic cells. At 3 days of MI (Fig. 2e), vimentin- (Fig. 2f) and α-SMA-positive (Fig. 2g) myofibroblasts expressed CRBP-1 (Fig. 2h). At 6 days after MI, numerous vimentin-positive fibroblasts (Fig. 2j) were also strongly positive for CRBP-1 (Fig. 2l). Certain subset of these fibroblasts was also positive for α-SMA (Fig. 2k). At 15 days of MI, the number of CRBP-1 positive cells was clearly decreased compared to 6 days (Fig. 2p). Higher magnification of photomicrographs for CRBP-1 positive cells was shown in the right two columns of Fig. 2. Part of CRPB-1 positive fibroblasts showed bizarre nuclei with prominent nucleolus at 3 days (Fig. 2e'). At 30 days, accumulation of fibroblasts was still visible (Fig. 2r). However, the number of CRBP-1-positive fibroblasts was diminished and CRBP-1 staining was weak (Fig. 2t); CRBP-1 was no longer detectable at 45 days after MI (data not shown). Alpha-SMA-positive and CRBP-1-positive cells were mainly distributed at the border between necrotic and viable myocardium, where the granulation tissue formation was prominent at 3 days and 6 days after infarction (Fig. 3-Ia-c). However, at 15 days and 30 days, a few CRBP-1-positive cells were observed for the most part at the center of the infarcted area, where numerous fibroblasts were identified. These areas were accompanied by a relatively small number of inflammatory cells. Double-labeling immunohistochemistry of CRBP-1 and α-SMA revealed that part of the CRBP-1-positive cells were α-SMA-positive myofibroblasts (Fig. 3-Id).

Morphometrical analysis revealed that α-SMA-positive area/cross-sectional area and CRBP-1-positve area/cross-sectional area were reached maximal at 6 days and decreased thereafter (Fig. 3-II). Western blot confirmed maximum expression of CRBP-1 at 6 days (Fig. 3-III). The majority of α-SMA-positive cells at 15 days, 30 days and 45 days following infarction were pericytes of capillaries, vascular smooth muscle cells of small arteries and thickened endocardium due to cardiac remodeling. We eliminated area of vessels and endocardium, in order to demonstrate the accurate ratio of occupied area by myofibroblasts per cross-sectional area.

Expression of MMP-2 and MMP-9 was not detected in the control hearts (Fig. 4a,b) and immune-reactive at 3 days (Fig. 4c,d). The density of these cells reached a maximum at 6 days (Fig. 4e,f) after infarction. Expression of MMPs

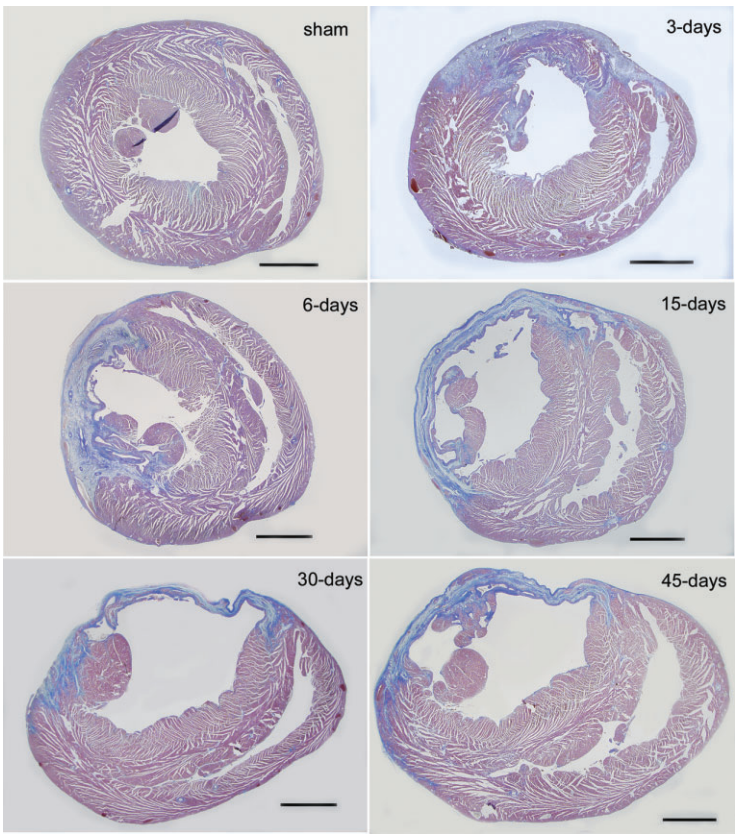

Figure 1 Cardiac remodeling after myocardial infarction in rat hearts. Loss of myocardium is evident at 3-days and transmural infarction is observed at 6-days after ligation of the left coronary artery. Thickness of the infarcted myocardial wall decreases from 15-days onward, while ventricular dilatation and myocardial hypertrophy increase. The infarcted region in the left ventricle contains few cardiomyocytes at 30-days, and scar tissue at 45-days. (Scale bar = 2 mm, Masson's trichrome).

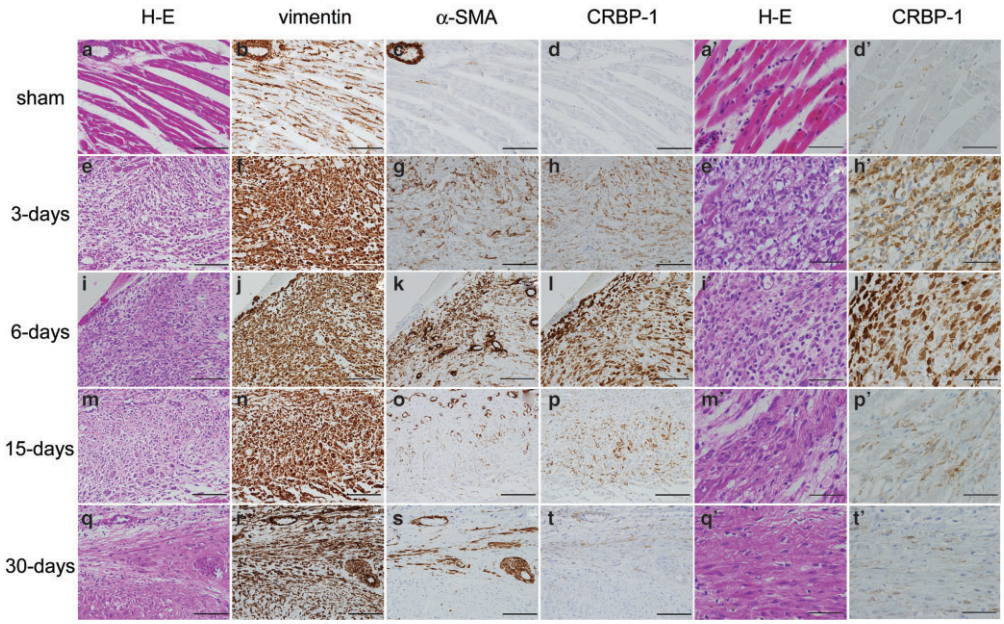

Figure 2 Transient expression of cellular retinol binding protein-1 (CRBP-1) in rat infarcted area. Sham operated animals show presence of vimentin-positive interstitial fibroblasts (**a** and **b**), whereas these fibroblasts are negative for α-smooth muscle actin (**c**) and CRBP-1 (**d**). At 3-days after infarction (**e**), vimentin- (**f**) and α-smooth muscle actin-positive (**g**) myofibroblasts clearly express CRBP-1 (**h**). At 6-days (**i**), vimentin-positive fibroblasts (**j**) strongly expressed CRBP-1 (**l**). These fibroblastic cells are partially positive for α-smooth muscle actin (**k**). At 15-day (**m**), proliferation of fibroblasts (**n**), including a few α-smooth muscle actin-positive myofibroblasts (**o**) are prominent; however, CRBP-1 positive fibroblasts are diminished (**p**). At 30-days after infarction (**q**), few CRBP-1-positive cells are observed (**t**), albeit presence of numerous fibroblastic cells (**r**) including myofibroblasts (**s**). Higher magnification of CRBP-1 positive cells was shown in right 2 columns (**a'**, **d'**, **e'**, **h'**, **i'**, **l'**, **m'**, **p'**, **q'**, **t'**). Part of CRPB-1 positive fibroblasts showed bizarre nuclei with prominent nucleolus at 3-days (**e'**). (a-t: Scale bar = 100 μm, higher magnification: Scale bar = 50 μm).

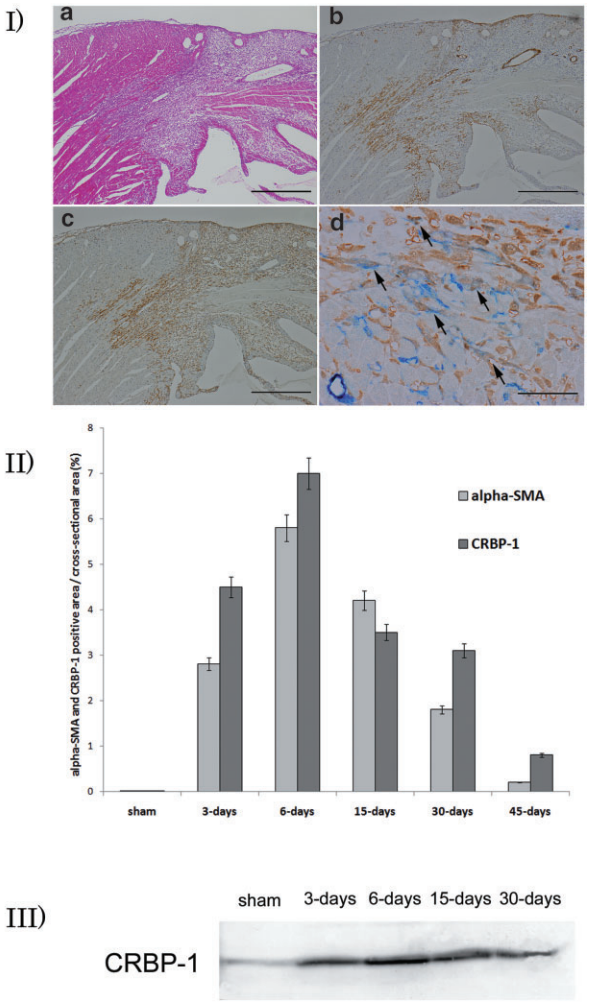

Figure 3 Photomicrograph of immunohistochemistry and double-labeling immunohistochemistry for α-smooth muscle actin (α-SMA) and cellular retinol binding protein-1 (CRBP-1), morphometrical analysis of immunohistochemistry, and Western blotting. Both α-SMA (**I-b**) and CRBP-1 (**I-c**) are distributed similarly at the border between viable and necrotic myocardium, where granulation tissue formation is prominent (**I-a**) at 3-days by lower magnification photomicrograph. Double-labeling immunohistochemistry for both α-SMA (**I-d**, blue) and CRBP-1 (**I-d**, brown) reveals that certain subset of CRBP-1-positive cells is myofibroblasts, as assessed by the presence of α -SMA (arrows). Expression of α-SMA and CRBP-1 reaches maximum at 6-days after infarction and decrease thereafter until 45-days by morphometrical analysis of immunohistochemistry (II). Western blotting reveals similar manner of CRBP-1 expression with morphometrical analysis (III). (I a-c: Scale bar = 500 μm, I d: Scale bar = 50 μm).

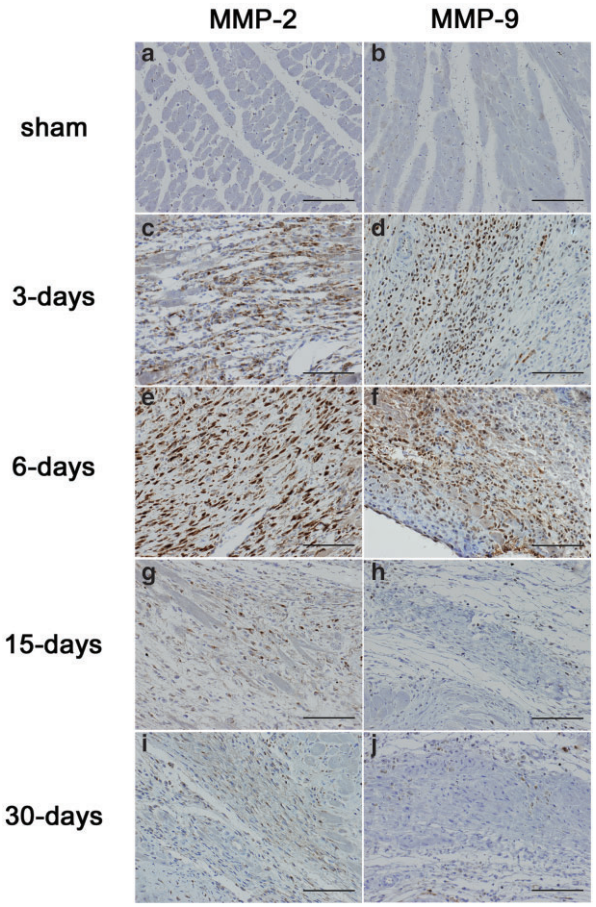

Figure 4 Immunohistochemistry for matrix metalloproteinase (MMP)-2 and MMP-9 in rat myocardial infarction. MMP-2 and MMP-9 expression was not detected in the control hearts (**a** and **b**). Thereafter, the density of both MMP-2 and MMP-9 expression increases at 3-days (**c**, **d**) and reaches maximum at 6-days (**e**, **f**) after infarction, which is similar to α-smooth muscle actin and cellular retinol binding protein-1 distribution. Expression of MMPs are gradually decreased at 15-days (**g**, **h**) and hardly detectable at 30-days (**i**, **j**). (a-j: Scale bar = 100 μm).

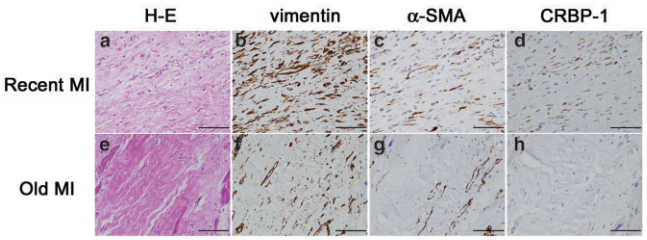

Figure 5 Cellular retinol binding protein-1 (CRBP-1) expression in human myocardial infarction. A seventy four years old male suffered myocardial infarction 1 month before death. Histological and immunohistochemical examination reveals that the infarcts area (**a**) contains fibroblastic cells infiltration (**b**), including myofibroblasts (**c**). A proportion of these cells express CRBP-1 (**d**). Myocardial scar tissue (**e**), obtained from a 78 years old male, who suffered myocardial infarction 1 year before death, is negative for CRBP-1 (**h**), despite presence of vimentin-positive (**f**) and α-smooth muscle actin-negative (**g**) fibroblasts. (a-h: Scale bar = 100 μm).

dramatically decreased at 15 days (Fig. 4g,h) and was hardly detectable at 30 days (Fig. 4i,j).

Human heart specimens from three cases of recent MI, five cases of old MI and five cases of normal myocardium were examined by immunohistochemistry. Sections of normal myocardium and acute MI were negative for CRBP-1 (data not shown). However, sections of recent MI (Fig. 5a) showed the presence of CRBP-1-positive fibroblastic cells (Fig. 5b,d),

a proportion of these cells was also positive for α-SMA (Fig. 5c). Scar tissue of old MI (Fig. 5e) contained a few vimentin-positive fibroblasts (Fig. 5f) without CRBP-1 expression (Fig. 5h).

DISCUSSION

CRBP-1 is transiently expressed in fibroblasts cultured from different organs *in vitro*.[27] During the healing of a skin wound[27] and during neointima formation after angioplasty in the arterial wall,[32] CRBP-1 is expressed by a subset of cells that appears early in the healing process and disappears, at least in part, through apoptosis. Most of these CRBP-1-positve cells in the neointima and in skin wound granulation tissue are myofibroblasts, as assessed by the presence of α-SMA.[27] This evidence suggests the possibility that CRBP-1 and RA are important factors in repair phenomena. Ventricular remodeling of the heart is a consequence of repair processes after MI. In the present rat model of left coronary artery ligation, LV dilatation develops during the first week after MI and continues to progress over 45 days. Increased RV weight and LV dimension of infarcted hearts implies that a certain degree of compensation has been reached through left ventricular remodeling. Ventricular wall remodeling appears to be a key determinant of clinical outcome in heart disease in general[31] and in post-MI recovery.[33] Such remodeling involves the production and destruction of extracellular matrix (ECM) proteins,[34] inflammation,[35] and apoptotic and/or necrotic cell death.[36] This is the first description that CRBP-1 is transiently expressed by fibroblastic cells during the process of cardiac remodeling after myocardial infarction.

Cardiac fibroblasts and myofibroblasts are crucially involved in remodeling processes through the production of growth factors and cytokines that act as autocrine and/or paracrine factors, as well as of ECM proteins and proteinases.[37] Synthesis and degradation of collagen during cardiac remodeling are mainly regulated by myofibroblasts. Moreover, myofibroblasts develop the force that is essential for connective tissue remodeling in response to mechanical stretch, vasoactive peptides, growth factors, and proinflammatory cytokines.[38,39] They are numerous in the granulation tissue of the infarct region and replace the lost myocardium by scar tissue. Interestingly, part of the CRBP-1-positive cells in our rat MI model at 3 days of infarction demonstrated oval cytoplasmic shape with bizarre nuclei accompanied by prominent nucleolus, suggesting immature fibroblasts for their pleomorphic appearance.

Some studies have suggested the possible effects of RA on cardiovascular remodeling.[40] Retinoic acid inhibits the angiotensin II induced smooth muscle cell proliferation by reducing angiotensin type I receptor expression.[41] Retinoic acid treatment reduces MMP production by cultured fibroblasts with attenuated degradation of ECM.[42] Furthermore, oral administration of all-trans RA significantly lowers plasma MMP-9 level in patients with emphysema.[43] Cells expressing CRBP-1 are more prone to respond to RA treatment than CRBP-1 negative cells.[44] In the current study, MMP-2 and MMP-9 expression demonstrated a similar tendency to CRBP-1 expression. These results suggest that CRBP-1 expression might be closely linked with synthesis and degradation of extracellular matrix during the cardiac remodeling after myocardial infarction. As cell proliferation and ECM degradation play an important role for LV remodeling, all the above described RA effects and our results might be related to the mechanisms of LV remodeling after MI. CRBP-1 regulates uptake, intracellular transport, and metabolism of RA, hence appears to be likely involved in these important functions.[45] Our observations suggest that CRBP-1 is involved in the early phases of active granulation tissue formation i.e. myofibroblast differentiation rather than contraction or collagen production. In our model, enhanced CRBP-1 expression corresponded with active granulation tissue, which is similar to human recent myocardial infarction at 2–4 weeks after onset. However, the relationship between CRBP-1 expression and tissue repair remain to be elucidated.

Left ventricle remodeling following MI is a major clinical problem that often leads to fibrosis, decreased performance, and congestive heart failure (CHF). The increase in the number of patients diagnosed with CHF highlights the need for therapeutic strategies, in addition to angiotensin-converting enzyme inhibitors and beta-adrenergic receptor blockers, in order to combat against the deleterious effects of LV remodeling. Loss of myocardium is progressive after coronary artery occlusion and this phenomenon is known as infarct expansion, which is a critical determinant of LV remodeling and thus of prognosis. New therapeutic strategies for connective tissue remodeling after MI should be targeted to the controlled modulation of the molecular and cellular factors involved in tissue repair. The effects of RA on myofibroblasts and on ECM might be one of the candidates for these activities.

In conclusion, we show that CRBP-1 is transiently expressed by fibroblast in a rat MI model and is expressed in the recent MI in humans. Our results suggest that CRBP-1 plays a relevant role in ventricular remodeling after MI, including myofibroblastic transformation and ECM metabolism, and may be useful in designing strategies to influence MI healing as well as in LV remodeling processes.

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