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Expression of the multifunctional Y-box protein, YB-1, in myofibroblasts of the infarcted rat heart

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Abstract

Intracellular signaling mechanisms regulating the turnover of α-SMA-positive myofibroblasts (myoFbs) at the site of myocardial infarction (MI) are poorly understood. Y-Box (YB)-1, a multifunctional protein, may be involved in regulation of proliferation, migration and apoptosis of myoFbs. Our objective was to study the expression of YB-1 in the infarcted rat heart and its localization in myoFbs. On days 3–28 following MI, we monitored YB-1 expression and its colocalization with α-SMA, and proliferation markers PCNA and Ki-67 in infarcted tissue by Western blot, immunohistochemistry, and immunofluorescent double-labeling. YB-1 is barely detectable in normal myocardium. At the infarct site, however, YB-1 is markedly elevated from day 3 post-MI concomitant with the induction of cell proliferation. MyoFbs are the major source of YB-1 and retain it up to day 28 post-MI. We suggest early expression of YB-1 promotes proliferation and migration of myoFbs, whereas prolonged expression may be responsible for scar formation.

Keywords: YB-1; Infarction; Remodeling; Myofibroblast; Proliferation

Wound healing after acute myocardial infarction (MI) is a highly complex and finely regulated process [1–3], which provides for the replacement of lost necrotic cardiomyocytes with a scar composed of fibrillar collagen. α-SMA-positive myofibroblasts (myoFb) are the key cells participating in the formation of the infarct scar. At the infarct site they first appear by days 3–5, actively proliferate and compose the dominant cell type after 2 weeks of MI [1,4,5]. The major function of myoFbs is elaboration of fibrillar collagen types I and III, stimulated by autocrine and paracrine secretion of various modulators of wound healing including renin, angiotensin II, and TGF-β [5–7]. In contrast to inflammatory cells, which appear and then disappear by apoptotic cell death by 2 weeks post-MI [8], myoFbs persist in scar tissue for prolonged periods of time [9]. Imbalance in proliferation, functioning, and apoptotic clearance of myoFbs may significantly affect wound healing [10]. Therefore, proper understanding of molecular mechanisms involved in the regulation of proliferation, activity, and survival of myoFbs at the infarct site may provide important clues for improved outcome and wound healing after myocardial infarction.

The aim of the present study was to establish a link between infarct myoFbs and the multifunctional transcription factor YB-1. Multiple studies have demonstrated a unique spectrum of activity of YB-1, ranging from regulation of cell proliferation and migration to cell survival and collagen synthesis [11–18]. YB-1 has been shown to upregulate several key proliferation proteins including DNA polymerase α [11,13], and cyclins A

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and B1 [15], as well as to downregulate growth arrest factor p53 [20]. YB-1 regulates the synthesis of type I collagen [16,17], activates the transcription of matrix metalloproteinase 2 (MMP-2) [18], increases the stability of renin mRNA [21], and down-regulates the pro-apoptotic gene Fas [19].

In this report we, for the first time, demonstrate induced expression of YB-1 after acute MI and its predominant localization in myoFbs found at the infarct site. Based on these observations we suggest that YB-1 might play an important role in the turnover of myofibroblasts at the infarct site.

Materials and methods

MI model. Myocardial infarction was induced by ligation of the anterior descending coronary artery in Sprague–Dawley male rats as previously reported [5]. Three rats with large infarctions (more than 20% of left ventricle circumference) for each time-point of MI and three unoperated rats serving as a control were used for our experiments. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Immunoblotting. Rats with MI were killed at 3, 5, 10, 14, and 28 days following ligation of the coronary artery. The infarcted tissue was cut from each left ventricle, frozen immediately in liquid N_2 and stored at −70 °C. For protein isolation the infarcted tissue was homogenized in the lysis buffer (7 M urea, 2 M thiourea, 0.8 % pharmalyte, pH 3–10, 2% Chaps, 1 mM PMSF, 1 mM leupeptin, and 1 mM aprotinin). After ultracentrifugation (33,000 rpm, 1 h at 4 °C using SW 50.1 rotor) samples were dialyzed overnight (0.02% KCl, 1.2% Tris, 10% glycerol, and 0.65% ethanol) and concentrated. Protein concentration was estimated by the Bradford method. Equivalent amounts of proteins from normal (50 μg) and infarcted hearts were boiled in sample buffer for 5 min, separated by 10–15% SDS–PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) with 5% non-fat dry milk for 2 h and then incubated with primary antibody overnight at 4 °C. Membranes were next washed in TBST, incubated with alkaline phosphatase-conjugated secondary goat anti-rabbit or goat anti-mouse antibody (Sigma, St. Louis, MO) at 1:5000 dilution for 1 h. Proteins were detected with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium. Anti-p53 (1:1000, clone BP53-12, Sigma) and anti-PCNA (1:1000 dilution, Ab1, Neomarker, Fremont, CA) antibodies were used. Polyclonal antibody to human YB-1 was obtained by immunization of rabbits with the recombinant protein expressed in *E. coli*. γ-Globulin fraction was isolated with ammonium sulfate precipitation (10 mg/ml) and used at 1:1000 dilution.

Immunohistochemistry and immunofluorescent microscopy. Hearts from unoperated and MI rats were embedded into OCT media and snap frozen at −70 °C. The same time-points of MI were used for immunohistochemistry as for Western blot. In addition 1-week-old MI hearts were used for certain immunofluorescent double-labeling because of the co-existence of a high level of cell proliferation and myoFbs at this particular time point.

Frozen tissue sections (6-μm) from unoperated control left ventricles and infarcted left ventricle were fixed in 10% buffered formalin for 10 min. Internal peroxidase activity was quenched with peroxidase blocking reagent (DAKO, Carpinteria, CA). Non-specific binding of antibodies was blocked with 5% heat-inactivated goat serum/3% BSA for 1 h. Primary antibodies for YB-1 (1:200), $p21^{WAF}$ (1:100, Ab5, Neomarker), anti-Ki-67 (1:50, BD Pharmingen, San Diego, CA), α-SMA (1:400, 1A4, Sigma) were applied in antibody diluent with background reducing components (DAKO) for 2 h at room temperature or overnight at 4 °C. Washing was performed after each step with PBS 3 × 5 min. Biotinylated secondary antibody and HRP-conjugated streptavidin (DAKO LSAB 2 System HRP) or HRP-conjugated goat anti-mouse antibody (1:150) (Sigma) was implemented. The target protein was visualized with DAB staining. Nuclei were counterstained with Mayer's hematoxylin. For negative control PBS or irrelevant antibody was used instead of the primary antibody (Fig. 2A).

For immunofluorescent double-labeling both primary antibodies were diluted with antibody diluent (DAKO) to reduce background intensity and applied together for 2 h at room temperature or overnight at 4 °C. Primary antibodies were used in the same dilutions as for immunohistochemistry. Rabbit p53 (1:200, Santa Cruz, CA) antibody was used to study co-localization with α-SMA. Washing was performed after each step with PBS three times for 5 min. Alexa Fluor-conjugated goat anti-rabbit (594 nm) and anti-mouse (448 nm) secondary antibodies (Molecular Probes, Eugene, Oregon) were applied in darkness for 1 h. The sections were mounted with Vectashield Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA) to counterstain nuclei. Images were acquired using AxioPlan2 microscope (Zeiss, Germany) and analyzed using Adobe Photoshop 7.0.

Isolation of myofibroblasts from infarcted heart and cell culture. For myofibroblast isolation, the tissue was excised from the infarct region of rat heart 1 week after left coronary artery ligation and placed into Hank's solution at 4 °C. The tissue was minced using two sterile scalpels, transferred to Hank's solution with 1000 U/ml collagenase II (270 U/mg), and placed in a shaker at 37 °C for 2 h. To ensure cell dispersion, the mix was passed through a 50-ml syringe several times and reincubated for an additional hour. The cells were washed with DMEM (Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Life Technologies); centrifuged (5 min at 1200 rpm); resuspended in 10% DMEM and plated onto 10-cm plates. Specificity of isolation was confirmed by staining with α-SMA and larger size of the cells when compared with rat aorta smooth muscle cells. For double-labeling immunofluorescence experiments the cells were trypsinized, plated on 35 mm coverslips and grown overnight. The next day cells were fixed with 10% buffered formalin and incubated for 2 h with primary antibodies in antibody diluent supplemented with 5% goat serum. After washing goat anti-rabbit Alexa Fluor 555 and anti-mouse Alexa Fluor 448 were added (1:500) for 45 min, washed with PBS and mounted with Vectashield Mounting Medium containing DAPI. Slides were viewed with AxioPlan2 microscope (Zeiss, Germany) and analyzed using Adobe Photoshop 7.0.

Results

YB-1 expression at the infarct site

YB-1 level is barely detectable in the normal heart. However, following MI a marked up-regulation of YB-1 protein on day 3 was observed and persisted up to day 10 post-MI (Fig. 1A). By day 28 we could not detect much YB-1 by immunoblotting (data not shown), although by immunohistochemistry we observed YB-1 expression up to 4 weeks post-MI (Fig. 2E). In agreement with the immunoblotting results, YB-1 was not found in the normal heart by immunohistochemistry (compare Fig. 2B with negative control 2A). In the infarcted hearts YB-1 was mainly expressed at the infarct site, and to a lesser extent in interstitial cells in

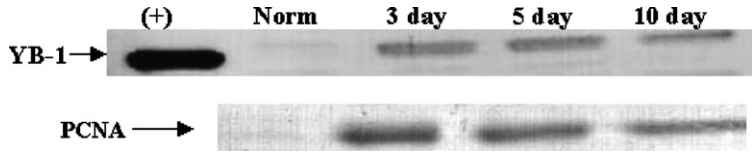

Fig. 1. Expression of YB-1 and PCNA in the infarcted tissue. Total protein was isolated from MI tissue at days 3, 5, and 10 (three rats for each time point) and subjected to immunoblotting analysis as described under Materials and methods. Purified YB-1 was used as a control in Western blots. Normal indicates protein isolated from control rat myocardium corresponding to the same area as that of MI.

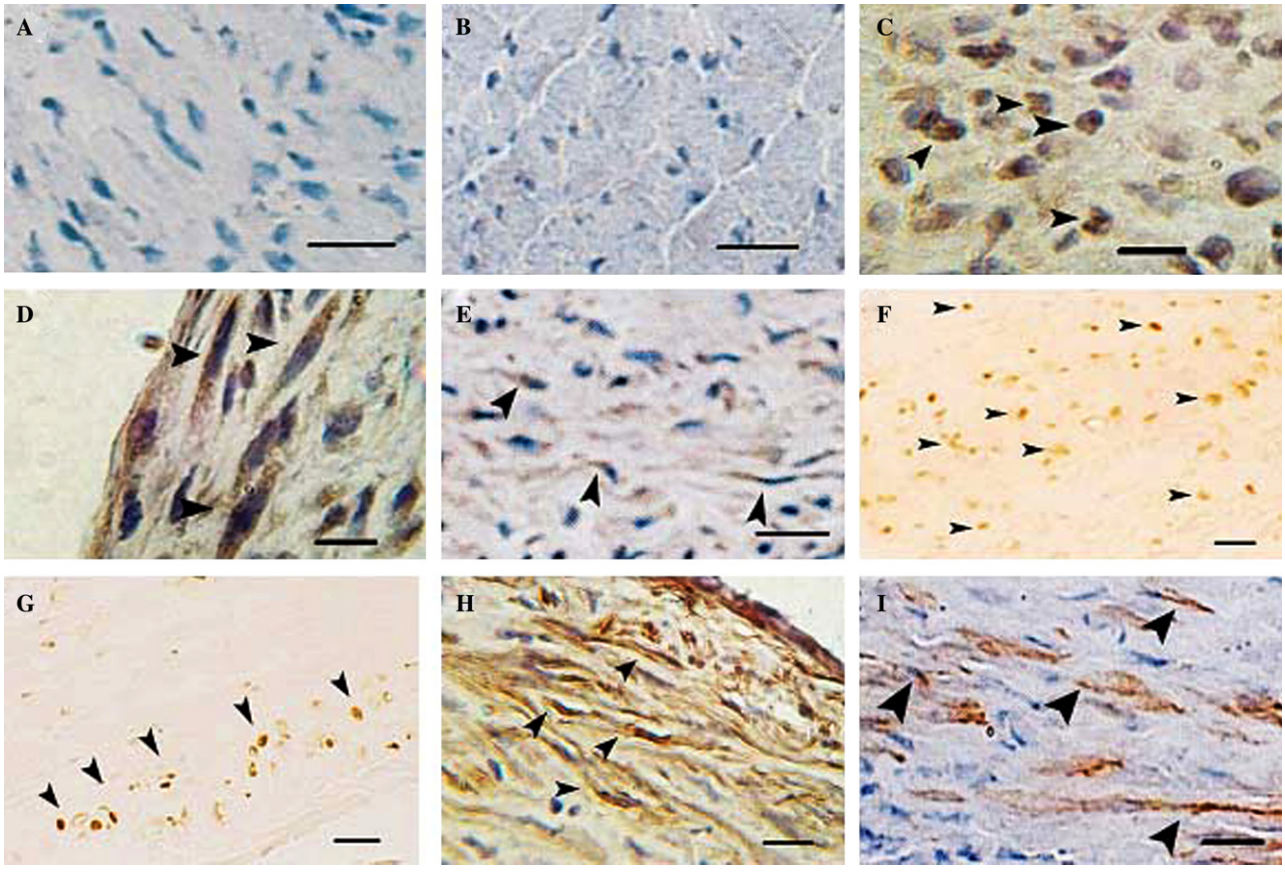

Fig. 2. Localization of YB-1, Ki-67, and α-SMA in the infarcted area by immunohistochemistry by DAB staining. Nuclei were counterstained with hematoxylin. (A) Negative control. (B–E) Staining for YB-1. (B) Normal heart; (C) MI 5 day; (D) MI 10 day; (E) MI 4 weeks. Arrowheads indicate YB-1-positive cells. Nuclear localization of YB-1 is seen on day 5, cytoplasmic YB-1 in myoFbs—on day 10 and 28 post-MI. (F,G) Staining for proliferation marker Ki-67 (without counterstaining). (F) MI 5 day; (G) MI 10 day. Arrowheads indicate Ki-67-positive proliferating cells (H,I) staining for α-SMA. (H) MI 10 day. (I) MI 4 weeks. Arrowheads indicate α-SMA-positive myoFbs. Scale bars: 10 μm.

the non-infarcted myocardium (Figs. 2C–E). In the early phase of MI healing (3–5 days) YB-1 was mainly localized in the nucleus (Fig. 2C). After day 10 YB-1 levels were considerably reduced and appeared to be predominantly localized in the cytoplasm (Figs. 2D and E). These results not only indicate YB-1 expression at the infarct site but also suggest compartmentalization, i.e., during the early phase of MI it is mainly localized in the nucleus whereas at later stages it is mainly localized in the cytoplasm.

YB-1 expression at the infarct site correlates with expression of the proliferation marker PCNA (Fig. 1B), i.e., it is undetectable in the normal heart but is significantly up regulated early after MI. In order to identify the cells expressing YB-1 we used antibody specific for Ki-67, a proliferation marker. Like YB-1, Ki-67 is highly expressed at the infarct site on day 3, peaks on the day 5, and gradually declined thereafter (Figs. 2F and G) to virtually undetectable levels by day 28 (data not shown). By double-labeling we found that the majority of Ki-67-positive cells express YB-1 (Figs. 3A–C). However, some elongated YB-1-expressing cells are Ki-67-negative (Fig. 3B), suggesting these may be differentiated myofibroblast cells, which retain YB-1

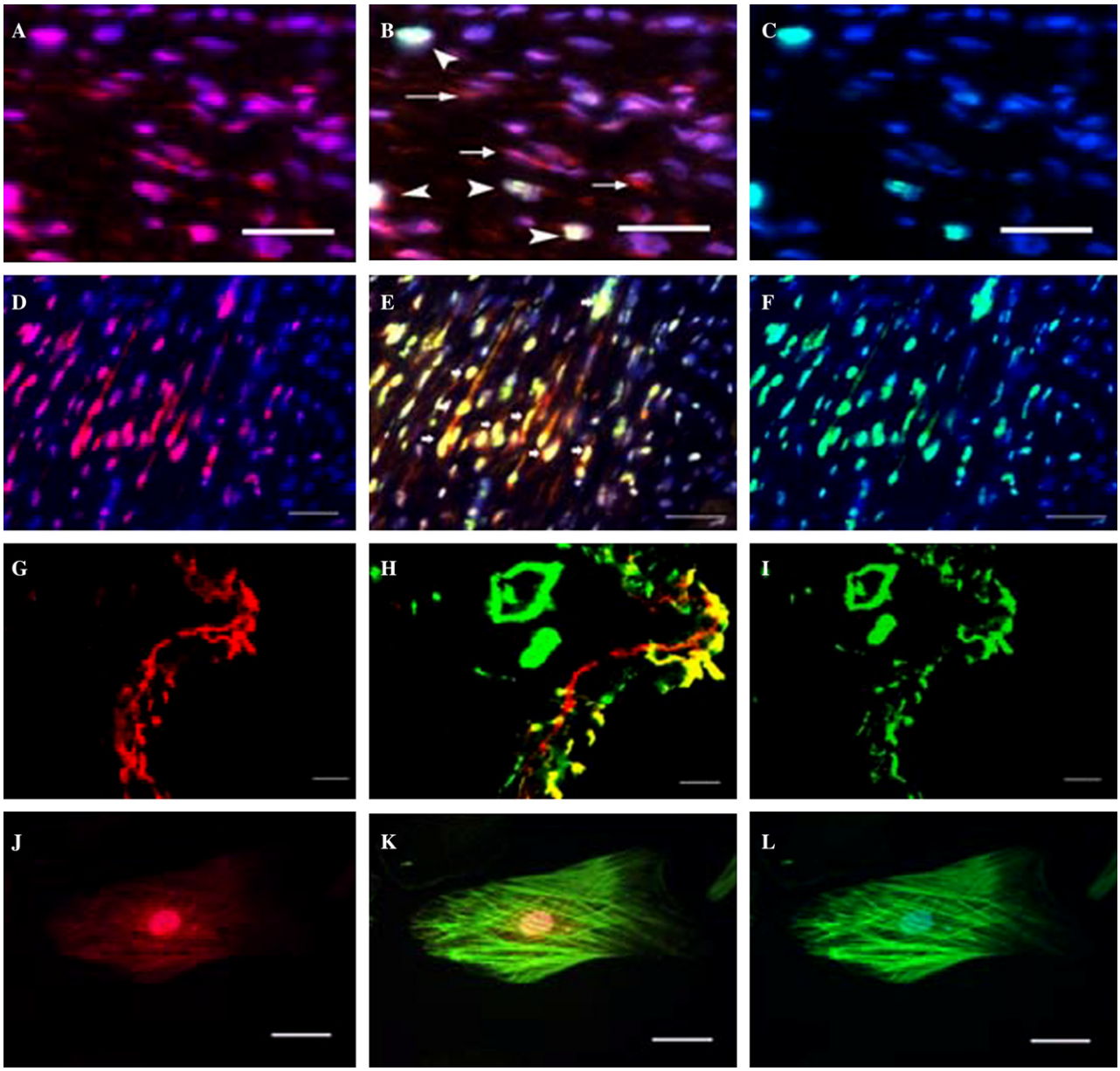

Fig. 3. Immunofluorescent double-labeling in the infarcted area. Nuclei stained with DAPI (blue). (A–C) Co-expression of YB-1 and KI-67 in the infarcted region at 1 week post-MI. (A) Staining for YB-1 (red); (C) staining for Ki-67 (green). Merged image of YB-1 and Ki-67. Yellow is the area of co-localization. Double-labeled cells are shown by arrowheads, only YB-1(+) cells—by arrows. (D–F) Co-expression of YB-1 and $p21^{WAF}$ at 2 weeks post-MI. (D) Staining for YB-1 (red); (F) staining for $p21^{WAF}$ (green); (E) merged image of YB-1 and $p21^{WAF}$. Double-labeled cells (yellow) are shown by arrowheads. (G–I) Co-expression of YB-1 and α-SMA in the infarcted region at 1 week post-MI. Subepicardial area. (G) Staining for YB-1 (red); (I) staining for α-SMA (green); (H) merged image of YB-1 and α-SMA. Yellow is the area of co-localization. (J–L) Co-expression of YB-1 and α-SMA in cultured myofibroblasts isolated from the infarcted area at 1 week after MI. (J) Staining for YB-1 (red); (L) staining for α-SMA (green); (K) merged image of YB-1 and α-SMA. Scale bars: 10 μm.

after growth arrest. To test this, we performed double-labeling of YB-1 with the growth arrest factor $p21^{WAF}$ (Figs. 3D–F) on day 14, when cell proliferation rate is already markedly diminished as shown by Ki-67 staining. Most of the cells on the day 14 of MI are $p21^{WAF}$-positive and also express YB-1. These results suggest YB-1 is also present in some non-proliferating, differentiated cells and further suggest other important functions in wound healing in addition to controlling cell proliferation.

α-SMA-positive myoFbs are the major cell type that provide an important function for wound healing after MI. MyoFbs appear at the MI site from 3 to day 5 [21] and remain there even around day 28 (Figs. 2H and I). To confirm that myoFbs express YB-1, we carried out double-labeling of YB-1 with α-SMA using

Alexa Fluor 594 and 448 dyes. As seen in Fig. 3H α-SMA-positive myoFbs widely express YB-1 (Figs. 3G–I). In contrast, vascular smooth muscle cells of the large coronary artery did not stain for YB-1, suggesting YB-1-positive α-SMA-positive cells are myoFbs (Fig. 3H).

YB-1 expression in cultured infarct myofibroblasts

In quiescent cells YB-1 was shown to be localized mostly in the cytoplasm whereas mitogenic stimulation induces translocation of YB-1 into the nucleus [11]. Nuclear YB-1 promotes initiation of cell proliferation by activating transcription of cell cycle regulatory proteins such as cyclins A and B and DNA polymerase α [11,13,15]. Our in vivo immunohistochemistry data show that at early stages of MI healing (3–5 days), when cell proliferation rate is highest, YB-1 is preferentially localized in the nuclei (Fig. 2C). MyoFbs constitute a major pool of proliferating cells in the infarcted zone [4], suggesting that nuclear YB-1-expressing cells include actively proliferating myofibroblasts. However, it was difficult to demonstrate in tissue sections by triple labeling of YB-1, Ki-67 and α-SMA, due to high non-specific binding of antibodies, autofluorescence and high cell density. In order to overcome this problem we isolated α-SMA-positive myofibroblasts from infarcted rat heart using collagenase II digestion. To enrich culture with dividing cells, myoFbs were plated at very low density and the majority of cells were found to proliferate as shown by Ki-67 staining (data not shown). Under these conditions we observed mostly nuclear and perinuclear localization of YB-1. An example is presented in Fig. 3 where strong nuclear staining for YB-1 is seen in the α-SMA-positive myofibroblast (Figs. 3J–L). These results clearly indicate nuclear accumulation of YB-1 in proliferating myoFbs and together with our in vivo data suggest a role for YB-1 in the proliferation of myofibroblasts at the site of MI.

Discussion

In this study, we focused on the expression, temporal response, and localization of the multifunctional protein YB-1, involved in the control of cell proliferation and survival [11–21], during the first 4 weeks that follow acute MI due to permanent coronary artery ligation. Our study led to several major findings: (i) YB-1, a major cell cycle regulating protein, is not expressed in normal myocardium but is markedly up regulated during healing of the infarcted myocardium; (ii) rapid induction of YB-1 parallels initiation of cell proliferation at the infarct site and correlates with the expression of the proliferation marker PCNA; (iii) at the infarct site YB-1 co-localizes with another proliferation marker Ki-67; and (iv) α-SMA-positive myofibroblasts are the major source of YB-1 at the infarct site.

YB-1 is mostly a cytoplasmic protein, which translocates into the nucleus upon mitogenic stimulation and activates transcription of several cell cycle regulatory proteins [11–13,15]. Inhibition of YB-1 with antisense oligonucleotides or siRNA down-regulates expression of DNA polymerase alpha and significantly inhibits cell proliferation [13]. Strong nuclear expression of YB-1 at an early time-point post-MI further supports a role for YB-1 in regulation of cell proliferation.

In this work we demonstrate that the majority of cells expressing YB-1 at the infarct site are myofibroblasts, which also constitute a major pool of proliferating cells in the infarcted zone [4]. We also show that cultured proliferating α-SMA-positive myofibroblasts isolated from the site of MI preferentially express YB-1 in the nucleus—a phenomenon observed in actively dividing cells [11]. Thus, strong expression of YB-1 in myoFbs and its co-localization with Ki-67 suggest a role for YB-1 in the regulation of myoFb proliferation in the healing infarcted tissue. YB-1 may also be involved in the migration of myoFbs at the infarct site through its activating effect on MMP-2 transcription [18].

Furthermore, we observed persistence of YB-1 in the myoFbs in fibrogenic phase of healing (days 14–28) when cell proliferation essentially ceases. In the fibrogenic phase of repair, Ang II and TGF-β drive synthesis of type I and III fibrillar collagens [5–7]. A high level of Ang II at the infarct site at 2–4 weeks is maintained by paracrine activity of myoFbs and their continuous expression of renin, ACE, and AT1 receptor [5,6]. Experimental data suggest both collagen and renin production may be regulated by YB-1. It has been shown that YB-1 activates TGF-β response element in the distal portion of the collagen α1(I) promoter [16]. Antisense oligonucleotides against YB-1 inhibit α1(I) procollagen promoter-driven transcription in cultured fibroblasts [17]. YB-1 also increases the half-life of renin mRNA, allowing for more renin to be synthesized [21]. Noteworthy, at 10–28 days YB-1 was predominantly localized in the cytoplasm of myoFbs supporting its role in mRNA turnover.

Thus, many functions attributable to YB-1—cell proliferation, regulation of type I collagen gene transcription, and regulation of renin synthesis on mRNA level—strongly suggest an important role for YB-1 in cardiac remodeling following MI.

Conclusions

Our findings allow us to hypothesize that YB-1 may play an important role in the regulation of turnover of myofibroblasts at the site of MI. In the early stage of

healing, up-regulation of YB-1 promotes rapid burst in cell proliferation. In the chronic phase of wound healing YB-1 may be involved in extracellular matrix remodeling by regulation of collagen and renin synthesis. Further studies directed toward inhibiting YB-1 are necessary to determine the mechanism of action for YB-1 in these various wound-healing events.

Acknowledgments

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