Virchows Arch (1997) 430:63–69

ORIGINAL ARTICLE

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In vivo responses of macrophages and myofibroblasts in the healing following isoproterenol-induced myocardial injury in rats

Received: 17 April 1996 / Accepted: 18 July 1996

Abstract To clarify the relation between macrophage and myofibroblast involvement in various myocardial diseases, the authors investigated the kinetics of these cells in the healing (scar tissue formation) following isoproterenol-induced myocardial injury in rats. Alpha-smooth muscle actin (α-SMA) expressing myofibroblasts were seen at the border of the affected area and appeared in the greatest numbers on days 3–7 post-injection, followed by a gradual decrease by day 35. The peak on day 3 was consistent with the timing of the highest proliferative activity of myofibroblasts. The number of ED1-positive macrophages began to increase as early as day 1, reaching a peak on day 3 within the injured myocardium. The expansion of ED1-positive macrophages preceded an increased number of α-SMA-positive myofibroblasts suggesting that myofibroblast proliferation and activation may be mediated by factors released by ED1-positive mcrophages in response to myocardial injury. The number of ED2-positive tissue-fixed, resident macrophages gradually increased from day 3 post-injection, and peaked on day 14, but the number of ED2-positive macrophages was consistently fewer than that of ED1-positive macrophages during the 35 day-observation period after the injection. The labelling index of the ED2-positive cells was maximal on day 14, indicative of local proliferation of resident macrophages. In the healing process after myocardial injury, ED1-positive macrophages increase markedly in the early stages; ED2-positive macrophages appear later.

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Key words Healing · Myocardial injury · Macrophage · Myofibroblast · Immunohistochemistry

Introduction

Myocardial injury leads to an increase in interstitial fibrosis or scar tissue and the healing and fibrous tissue response following myocardial injury involves inflammatory cell infiltrates and extracellular matrix accumulation. Recent studies have indicated that fibroblast activation and proliferation are responsible for the accumulation of extracellular matrix proteins in the injured myocardium [10, 11, 19]. Macrophages have been known to be present in fibrotic areas for a long time [24], and their roles in the remodelling process and fibrogenesis have been assessed in different tissues. In vitro observations have demonstrated that macrophages produce highly fibrogenic growth factors, transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF) [23, 26, 28]. These growth factors may induce phenotypic modulation of fibroblasts towards myofibroblast-like cells, culminating in increased production of extracellular matrix proteins [30]. Accordingly, it has been suggested that macrophages may play a pivotal role in the fibrogenic response in the healing process in vivo after myocardial injury.

The presence of myofibroblasts, which were identified immunohistochemically by detection of cytoplasmic α-smooth muscle actin (α-SMA), has been reported in fibrogenesis and scar tissue in various pathological settings [2, 6, 21, 25, 34, 37–39]. The cells derived from granulation tissue fibroblasts are intermediate in nature between fibroblasts and smooth muscle cells and relate to tissue contraction [6, 30, 34]. Although α-SMA expressing myofibroblasts have been observed in the fibrous tissue following myocardial injury [37–39] and in the interstitium of experimentally induced myocardial hypertrophy [25], the relationship between macrophages and myofibroblasts in the course of the healing and fibrogenesis in the heart remains to be elucidated.

In the present study, the authors investigated the kinetics of macrophages and myofibroblasts immunohistochemically during the development of scar tissue following isoproterenol-induced myocardial injury in the rat. Administration of isoproterenol, a β-adrenergic stimulant, produces myocyte necrosis due to local ischaemia, leading to granulation tissue and scar. This experimental system has been already used as a model for studies of myocardial injury [29].

Materials and methods

A total of 33 male Sprague-Dawley rats (Clea Japan, Japan), weighing 220–260 g, were used. Twenty-four animals were given a single subcutaneous injection of isoproterenol; the dose was 10 mg/kg body weight in 1.0 ml saline solution. Three animals were sacrificed under ether anaesthesia at 12 h, 24 h, 3, 5, 7, 14, 21 and 35 days after the injection, respectively. The remaining 9 that received only saline solution, 3 each, were killed immediately (day 0), 7 and 35 days after the injection, and served as controls. A standard laboratory diet and water were available ad libitum throughout the experiment. To investigate the proliferating cell activity, each animal received a single intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma, UK), 100 mg/kg body weight, 1 h prior to sacrifice. After fixation in B5 solution containing mercuric chloride for 5 h [21], transverse slices of the heart including both ventricles were embedded in paraffin. Serial sections at 3 μm in thickness were mounted on poly-L-lysine coated slides, and stained with haematoxylin and eosin, and Masson's trichrome.

For immunohistochemical staining, tissue sections were deparaffinized, desublimated and washed in phosphate-buffered saline (PBS). After treatment with 0.1% trypsin solution in PBS for 10 min at 37° C, endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 5 min at room temperature. The slides were blocked with 10% goat serum in PBS for 20 min at room temperature and then incubated overnight at 4° C with mouse monoclonal antibodies to ED1 (Chemicon, USA) diluted 1:800, ED2 (Serotec, UK) diluted 1:400, α-SMA (DAKO Japan, Japan) diluted 1:800, and with a rabbit polyclonal antibody to von Willebrand factor (vWF; DAKO) diluted 1:1,800. Subsequently, goat anti-mouse or anti-rabbit biotinylated secondary antibody (DAKO) diluted 1:800 was applied for 1 h at room temperature, followed by incubation with streptavidin-peroxidase complex (DAKO) for 20 min at room temperature. After rinsing in PBS, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as a substrate for visualization and the sections were counterstained with haematoxylin. Slides processed with non-immune mouse or rabbit sera instead of primary antibodies served as negative controls. ED1 recognizes monocytes and newly recruited, as well as resident, macrophages. ED2 recognizes tissue-fixed, resident macrophages, such as Kupffer cells in the liver [9]. The value of ED1 and ED2 for the identification of macrophages in the rat has been established previously [9, 17]. Anti-α-SMA recognizes a subclass of myofibroblasts as well as smooth muscle cells [33]. The antibody to vWF is also used as an endothelial cell marker [31].

To investigate nuclear incorporation of BrdU in α-SMA-positive myofibroblasts or ED2-positive macrophages, double-labelling immunohistochemical stainings were performed [22]. As described above, sections were first reacted with anti-α-SMA or ED2, and incubated with secondary biotinylated antibody and with streptavidin complex. They were immersed in DAB to yield a brown reaction product. Subsequently, after treatment with 4 N hydrochloric acid for 30 min at 37° C to denature DNA, the slides were reacted with anti-BrdU mouse monoclonal antibody (DAKO) diluted 1:50 for 1 h at room temperature. Then they were incubated with peroxidase-conjugated anti-mouse IgG FC fragment antibody (Jackson Immunoresearch Laboratories, USA) for 1.5 h at 37° C, followed by black visualization with nickel-modified DAB.

Immunoreactive cell numbers were counted in five randomly selected different fields of the affected area at a magnification of ×400 using an eyepiece graticle. The mean value per unit area (0.1 mm^2) was calculated from the total numbers of labelled cells in three animals at each time point examined. For assessment of ED1- and ED2-positive cells, only those that had been sectioned through the plane of the nucleus were counted. Alpha-SMA- and vWF-positive cells were counted when a nucleus and at least one cytoplasmic process were present. Labelling indices were obtained for proliferating cells in double-labelled cells as a percentage of the total number of ED2- and α-SMA-positive cells, respectively; 200–500 cells were counted for each animal. The results were expressed as mean±standard deviation. Since no differences in the immunoreactive cell number were observed among three control groups (days 0, 7 and 35), they were regarded as being one group for statistical analysis. Cell counts in the treated animals were compared with those of the control group using Student's *t*-test; $P<0.05$ was considered significant.

Results

Isoproterenol-induced myocardial injury was characterized by multifocal lesions mostly in the subendocardial portions of left ventricle. These changes were generally similar to those described previously [29]. On day 1 (12 and 24 h) after the injection, necrotic myocytes were observed. Inflammatory cells, which consisted mainly of neutrophils and macrophages, began to accumulate around the affected area. On days 3 and 5, macrophages phagocytizing cell debris were frequently observed; a reparative process was seen in the viable myocardium at the edge of the lesion and appeared to progress toward its centre. On days 7, 14 and 21, granulation tissue, which consisted of fibroblasts, vascular endothelial cells and inflammatory cells, developed in the affected area. The cellularity in the lesion peaked on day 7, and thereafter diminished on days 14 and 21, accompanied by an increase in collagen fibres demonstrable by Masson's trichrome stain. On day 35, the lesions had almost healed with formation of scar tissue.

In control animals, ED1 and ED2 immunoreactivities were detected in spindle-shaped interstitial cells. The immunolabelled cells also showed a regular network of interstitial cells throughout the cardiac tissue; they were regarded as tissue-fixed, resident macrophages. No noticeable changes in localization of ED1 and ED2 immunoreactive cells were seen among control rats killed on days 0, 7 and 35. The number of ED1- and ED2-positive cells per 0.1 mm^2 in the normal myocardium was 5.5±2.3 and 6.3±2.5, respectively.

In rats injected with isoproterenol, there was an expansion of ED1-positive cell population within and around the affected myocardium. The kinetics of ED1-positive cells is shown in Fig. 1. The cell number significantly increased as early as 12 h, reaching a peak on day 3. Most of the immunoreactive cells during this phase resembled infiltrating blood monocytes and activated macrophages, morphologically phagocytizing cell debris (Fig. 2A). On days 5 and 7, the cell number decreased rapidly but remained abundant in the granulation tissue. On day 14, a slight increase in the ED1-positive cell

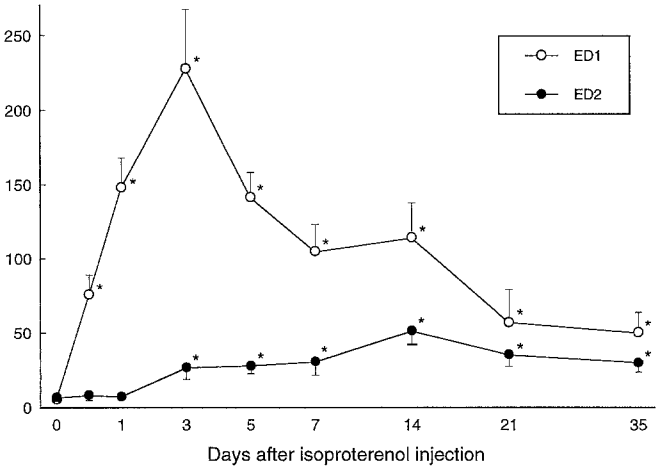


Fig. 1 The kinetics of ED1- and ED2-positive cell numbers in the rat myocardium following isoproterenol injection. Each value represents mean±standard deviation. The asterisks indicate values significantly different from control values, $P<0.01$. Values on day 0 are representative of control groups, because there was no difference amongst values on days 0, 7 and 35

Table 1 Proliferative activity for ED2- and α-smooth muscle actin (α-SMA)-positive cells following the isoproterenol injection (*BrdU* bromodeoxyuridine, *NE* not examined)

Days after injection	Labelling indices[a]	
	BrdU/ED2	BrdU/α-SMA
1	1.1±0.2	NE
3	0.9±0.3	8.4±2.5
5	1.3±0.7	4.4±0.7
7	2.8±0.9	2.4±0.5
14	3.3±0.8	1.1±0.6
21	2.5±0.5	0.0±0.0
35	1.4±0.4	NE

[a] Labelling indices mean the percentage of BrdU labelled cells in the total number of α-SMA- and ED2-positive cells, respectively. Each value represents mean±standard deviation of three animals

number was seen with the second peak. Although the cell number gradually decreased thereafter, it did not return to control level by day 35. On day 14 and subsequent days, granular cytoplasmic ED1-positivity was present in small round cells; in addition, especially at the border zone of the affected tissue, occasional ED1-positive cells were spindle in shape, giving an appearance of dendritic cells or resident macrophages, and the reactivity was also present in Anitsuchkow cells with their characteristic configuration of nuclear chromatin (Fig. 3A). In contrast, the kinetics of ED2-positive cells was different (Fig. 1). No significant change in this cell population was seen by 24 h post-injection. A gradual increase in the ED2-positive cell number was seen on days 3, 5 and 7, and reached a peak on day 14 (Fig. 4). Thereafter, the cell counts subsided but remained greater than the control level by day 35 (Fig. 1). Although the ED2-positivity appeared in large, round- or oval-shaped cells in the fibrotic area on day 14 and subsequent days, the reactivity was also present in spindle-shaped Anitschkow cells (Fig. 3B). Throughout the observation period, the number of ED2-positive cells was considerably fewer than that of ED1-positive cells.

In control animals, a few double-labelled cells for ED2 and BrdU were detectable in the interstitium. In the treated rats, the double-labelled cells appeared in the affected area (Fig. 5). The labelling index showed a peak on day 14 and thereafter fell towards the control value by day 35 (Table 1).

Alpha-SMA immunoreactivity in the heart of controls was restricted to smooth muscle cells constituting blood vessels. At 24 h post-injection, no immunoreactive cells for α-SMA were detected in the affected area. On days 3–7, however, there was a dramatic increase in the number of nonvascular α-SMA-positive cells (Fig. 6); they were seen predominantly in the periphery of the newly formed granulation tissue. Alpha-SMA-positive myofibroblasts were spindle-shaped with an oval nucleus, and larger in size than normal fibroblasts. In addition, the positive cells seen at the border of the affected area reacted more intensely for α-SMA, and were arranged in parallel to the longitudinal axis of the intact cardiac fibre (Fig. 2B). The positive reactions appeared filamentous. On subsequent days 14 and 21, the number of α-SMA-positive cells apparently decreased (Fig. 6), and the immunoreactive cells were occasionally observed in scar tissue on day 35.

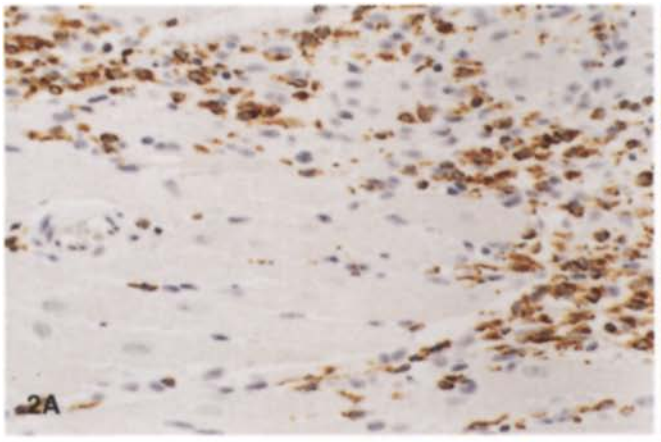
A few double-labelled cells for α-SMA and BrdU were observed in smooth muscle cells forming the vessels in control animals. In treated rats, the double-labelled cells were increased at the border of the injured area, indicating active proliferation of myofibroblasts (Fig. 7). The labelling index for the proliferative activity was maximum on day 3, and thereafter declined. No double-labelling cells were detectable on day 21 (Table 1).

In control animals, the antibody against vWF stained endothelia lining the large vessels and endocardium intensely. In contrast, capillary endothelial cells were slightly positive for vWF. Following the isoproterenol injection, vWF immunoreactivity was seen in neovascularized capillaries in the granulation tissue; the number of vWF-positive cells reached a peak on day 3, followed by a gradual fall. On day 35, there were still a small number of vWF-positive capillaries in the scar tissue (Fig. 6).

Discussion

The administration of isoproterenol to rats induced myocardial necrosis, followed by an acute inflammatory response and phagocytosis of necrotic debris. This necrotic lesion was replaced by granulation tissue and later by fibrocollagenous scar tissue. Cellular events in the healing of the isoproterenol-induced lesions bore a close resemblance to those of previous studies [29] and of those seen in other myocardial injury including infarction [12, 38].

In dermal wound healing [6, 34], myocaridal repair [37, 38], and pulmonary and liver fibrosis [2, 21], myofi-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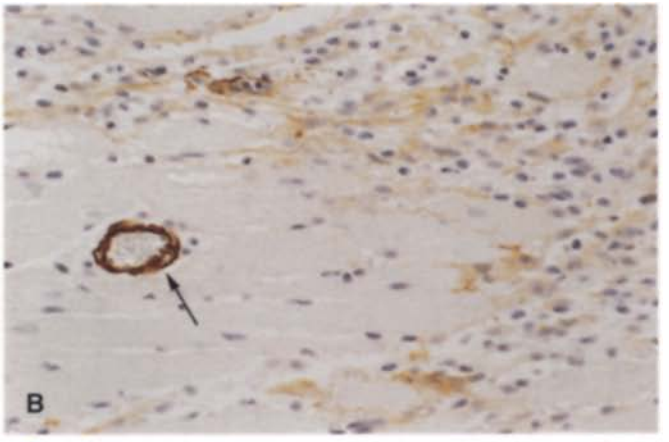
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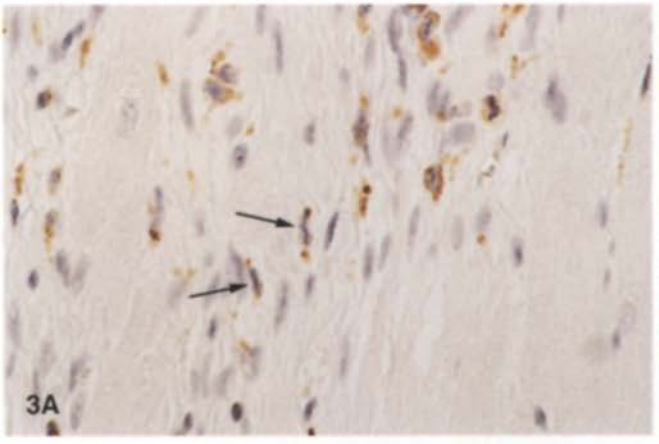
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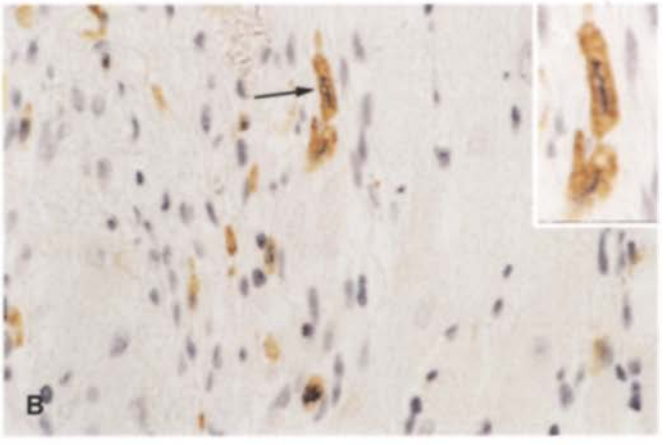
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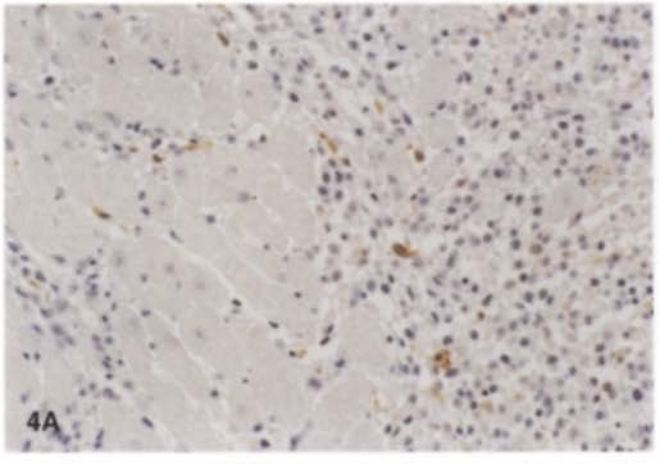
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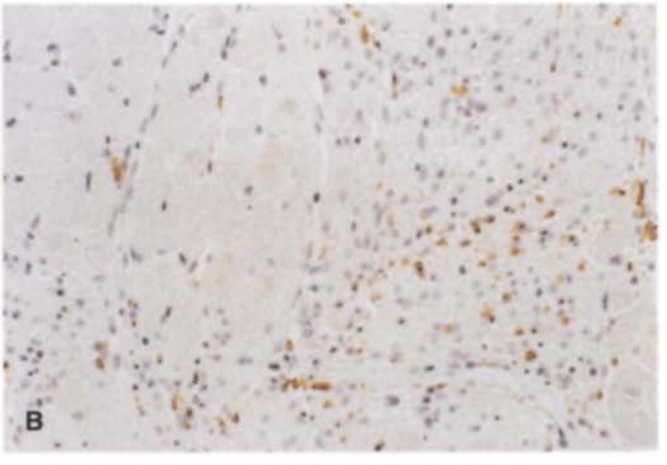
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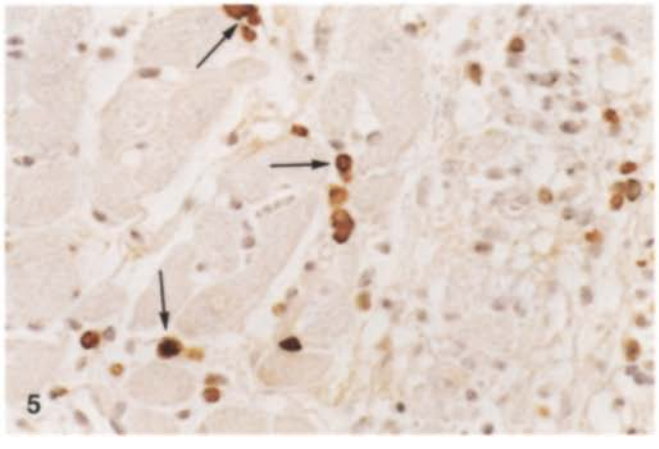
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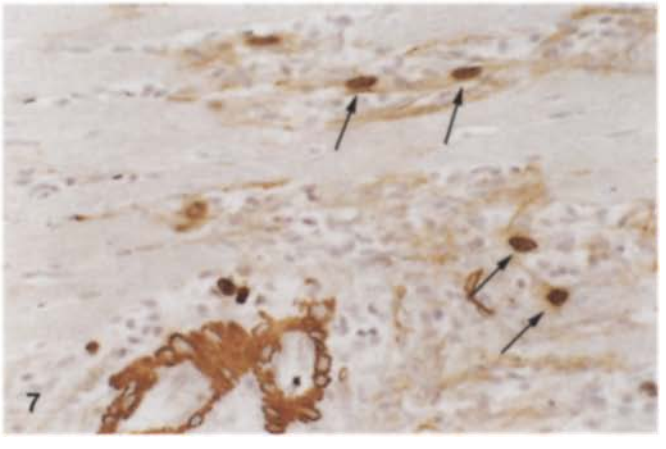
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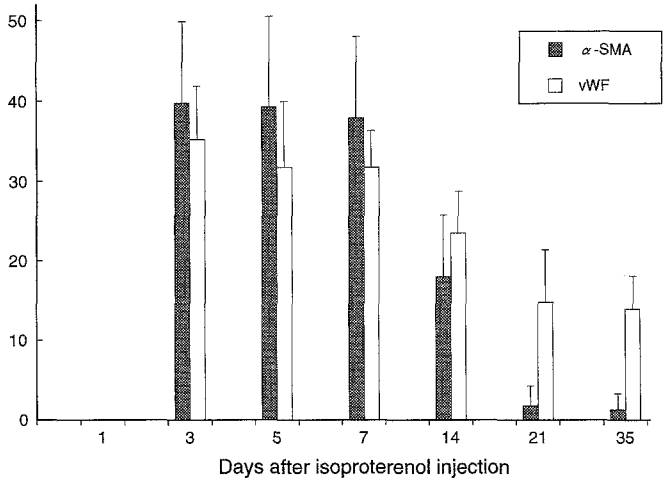

Fig. 6 Alpha-SMA- and von Willebrand factor -positive cell numbers in the rat myocardium following isoproterenol injection. Each column represents mean±standard deviation

broblasts have been observed. The cells were identified by ultrastructural features with abundant rough endoplasmic reticulum and cytoplasmic myofilaments [30], and by immunohistochemical detection of cytoplasmic α-SMA [13, 33]. In experimentally-induced rat myocardial infarction, Vracko et al. [37] have shown that α-SMA expressing myofibroblasts were identified as early as day 2 and were most prominent on day 4, but no quantitative analysis was performed. In the present study, the number of α-SMA-positive myofibroblasts increased dramatically on day 3 post-injection, and the largest number was retained by day 7; the cell number decreased thereafter with scar tissue formation. The double-labelling immunohistochemical staining revealed that the labelling index for α-SMA and BrdU peaked on day 3, in conformity with a dramatic increase in α-SMA-positive cell number. These findings suggest that the expansion of myofibroblast population was due, at least in part, to local proliferation. The transient proliferation of myofibroblasts in the myocardial healing process was in agreement with previous reports [6, 37, 39].

The emergence of myofibroblasts in tissue injury has been postulated as a mechanism for effecting tissue contraction [30]. The origin of myofibroblasts in the heart and elsewhere is unknown but recent studies have shown that various peptide growth factors may act to produce phenotypic modulation of fibroblasts [7, 27, 30], endothelial cells [3] or liver perisinusoidal cells [4] into myofibroblast-like cells. Desmoulière et al. [8] proposed TGF-β1 as the key molecule for the induction of α-SMA expression in cultured fibroblasts from subcutaneous granulation tissue in rats. A more complete quantitative analysis and additional studies of the cytoskeletal protein profile of myofibroblasts may provide important clues with regard to their origin [34].

Activated macrophages have been known to produce various growth factors such as TGF-β1, PDGF and tumour necrosis factor in the development of scar tissue [23]. It has been recently demonstrated that a number of α-SMA expressing perisinusoidal cells markedly increased following an expansion of ED1-positive macrophage population in chemically- and cholestatic-induced acute liver injury [18, 21]. In these studies, it was presumed that the perisinusoidal cells may be modulated to myofibroblastic cells by macrophage-derived growth factors, resulting in the production of matrix proteins [18, 21]. In the present study, we showed that a markedly increased number of ED1-positive macrophage occurred within the affected tissue immediately after the injection, reaching a peak on day 3. This was followed by a significant increase in α-SMA expressing myofibroblasts on days 3–7 when granulation tissues began to be formed.

ED1 is specific to circulating population of monocytes or macrophages. A marked increase in number of ED1-positive cells in injured or pathological tissues was regarded to be a result of migration into the injured site from circulating system [9, 17]. Thus, recruitment of blood monocytes might have contributed to the expansion of macrophage population in the injured myocardium. The present findings also suggest that blood monocyte-derived macrophage influx in response to myocardial injury may be involved in mediating myofibroblast induction and proliferation, perhaps through products released by macrophages, as suggested in other pathological conditions [18, 21].

Fig. 2A, B Rat myocardium on day 3 after isoproterenol injection. **A** Immunostaining for ED1. Many ED1-positive macrophages are seen; they resemble infiltrating blood monocytes and activated macrophages morphologically (×250). **B** Immunostaining for α-smooth muscle actin (α-SMA). α-SMA-positive myofibroblasts are seen at the edge of the ganulation tissue. α-SMA reactivity is intense in the smooth muscle of blood vessels (*arrow*; ×250)

Fig. 3A, B Rat myocardium on day 14 after isoproterenol injection. **A** Immunostaining for ED1. ED1-positive macrophages are seen in fibrotic area, showing a granular staining pattern of the cytoplasm. As compared with ED1-positive cells shown in Figure 2A, the cells are spindle in shape. Some positive cells (*arrows*) have a caterpillar-like configuration of the nuclear chromatin, giving the appearance of Anitschkow cells (×400). **B** Immunostaining for ED2. ED2-positive cells are present in the fibrotic tissue, showing a diffuse staining pattern for the membrane antigen. ED2 recognizes tissue-fixed, resident macrophages. A few ED2-positive cells (*arrow*) also have a caterpillar-like configuration of the nuclear chromatin (×400). *Inset* higher power view of the caterpillar-like cells (×690)

Fig. 4 Immunostaining for ED2 in the rat myocardium on days 3 (**A**) and 14 (**B**) after isoproterenol injection. **A** A few ED2-positive macrophages are present in the granulation tissue (×250). **B** Note the increase in number of ED2-positive macrophages in the fibrotic area (×250)

Fig. 5 Rat myocardium on day 7 after isoproterenol injection. Double-labelling method for ED2 and bromodeoxyuridine (BrdU). In the injured area, double-labelled cells (*arrows*) show simultaneous cytoplasmic ED2-positive, brown-staining and nuclear BrdU-positive, black-staining, indicating tissue-fixed, resident macrophages in S phase (×400)

Fig. 7 Rat myocardium on day 3 after isoproterenol injection. Double-labelling method for α-SMA and BrdU. In the injured area, double-labelled cells (*arrows*) show simultaneous cytoplasmic filamentous α-SMA-positive, brown-staining and nuclear BrdU-positive, black-staining, indicating active proliferation of myofibroblasts (×250)

An another interesting observation was the orientation and localization of myofibroblasts. At the border of the affected tissue, the majority of myofibroblasts were oriented parallel to intact adjacent myocytes; these cells reacted intensely to α-SMA. It has also been reported that myofibroblasts in scar tissue showed a parallel orientation to surviving myocytes in myocardial infarction in rats [38] and humans [39]. The reason for this orientation in the scar tissue is not clear, but a possible explanation for preferential alignment in myocardial scars is the continuous mechanical stress caused by the ongoing contraction and relaxation of myocardium [39].

Thompson et al. [36] have shown that, in experimental rat myocardial infarction, strong immunoreactivity and increased mRNA level of TGF-β1 were found in myocytes at the margin of the infarcted area. TGF-β1 derived from myocytes may play a causative role in the localization of myofibroblasts at the border site, in addition to that derived from macrophages. It is interesting to note that the kinetics of vWF-expressing neovascularized capillaries was generally similar to that of myofibroblasts; the number of vWF-positive capillary endothelial cells in granulation tissue was preceded by an expansion of ED1-positive macrophages. This would support the previous report that infiltrating macrophages are a possible stimulus for angiogenesis in wound healing [20].

In agreement with the second peak in the ED1-positive cell number, the number of ED2-positive cells was maximal on day 14. Moreover, local proliferation of ED2-positive cells was demonstrated by the double-labelling methods; the labelling index also peaked on day 14. Besides newly recruited monocyte-derived macrophages, ED1 antibody recognizes tissue-fixed, resident macrophages including dendritic cells which are considered to be a subpopulation of resident macrophages [9, 35]. ED2 antibody is highly specific for resident macrophages [9]. Zhang et al. [41] have demonstrated that an increased number of dendritic cells was maximal at the border zone between normal and necrotic tissue 7–14 days after coronary ligation. The second peak in ED1-positive macrophages in the present study might reflect the increase of both dendritic cell population and other resident macrophages recognized by ED2 antibody.

Dendritic cells have been considered to be mobilized from bone marrow in response to injury [41]. ED2-positive tissue-fixed, resident macrophages reside in major tissues throughout the body where they can be stimulated to proliferate after onset of disease or injury [9, 15, 35]. Except for the Kupffer cells in the liver [18, 21] and interstitial macrophages in the lung [1], the role of resident macrophages in the development of fibrosis has not been investigated. Dendritic cells have an important function on the initiation of immune responses, because they participate in antigen presentation and in the activation of immune effector cells [16]. It has also been shown that the dendritic cells functionally influence not only T lymphocytes but ED2-positive resident macrophages in infarcted [41] and hypertrophied rat heart [40]. ED2-positive resident macrophages provide processed antigen fragments for the antigen-presenting dendritic cells through their phagocytic activity [41]. This implies that ED2-positive cells have a potential to act as immune effector cells in the chronic inflammatory state of the myocardial healing process. Macrophages can change their phenotypic and functional characteristics depending on sites, maturation and state of activation [14]. It is possible that circulating monocytes enter the injured tissue and differentiate into macrophages expressing ED2 antigen with the development of scar tissues [5]. The repertoire of cytokines secreted by ED2-positive resident macrophages, which may contribute to the regulation of the healing process, remains to be clarified.

On day 14 and thereafter, ED1- and ED2-positive cells with their characteristic caterpillar-like configuration of nuclear chromatin were seen in the fibrotic tissue. They morphologically resembled Anitschkow myocytes which have been observed frequently at the border of the healing infarct [12, 29]. They are also seen in rheumatic fever in humans and in some animals in association with Aschoff bodies [32]. The origin of these cells is still unknown, but myocytes, nerves, histiocytes, endothelial cells and fibroblasts have been proposed as possible precursors [12]. Our findings suggest that the Anitschkow myocyte is a subtype of resident macrophages or is at least derived from the monocyte-macrophage lineage.

We have demonstrated quantitative data on the kinetics of macrophages and myofibroblasts during the healing of myocardial injury. Proliferation of α-SMA-expressing myofibroblasts is preceded by an expansion of the macrophage population. ED1-positive recruited macrophages and ED2-positive tissue-fixed, resident macrophages differ in the time of their appearance in the healing process; the former are prevalent in the early inflammation and the latter in the later stages.

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