American Journal of Pathology, Vol. 147, No. 2, August 1995

Collagen Remodeling after Myocardial Infarction in the Rat Heart

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In this study changes in the amount and distribution of types I and III collagen mRNA and protein were investigated in the rat heart after induction of a left ventricular myocardial infarction (MI). Sham operated rats served as controls. The animals were sacrificed at different time intervals after operation. Northern blotting of cardiac RNA and hybridization with cDNA probes for types I and III procollagen revealed a 5- to 15-fold increase in the infarcted left ventricle. Type III procollagen mRNA levels were already increased at day 2 after MI, whereas type I procollagen mRNA followed this response at day 4 after MI. This increase was sustained for at least 21 days in the infarcted left ventricle for type III procollagen mRNA, whereas type I procollagen mRNA levels were still elevated at 90 days after MI. In the noninfarcted right ventricle a 5- to 7-fold increase was observed for both type I and type III procollagen mRNA levels, but only at day 4 after MI. In the non-infarcted septum a transient increase was observed for type I procollagen mRNA from day 7–21 (4- to 5-fold increase) and a decline to sham levels thereafter. In the septum type III procollagen mRNA levels were only elevated at 7 days after MI (4- to 5-fold increase) compared with sham operated controls. In situ hybridization with the same types I and III procollagen probes showed procollagen mRNA-producing cells in the infarcted area around necrotic cardiomyocytes, and in the interstitial cells in the non-infarcted part of the myocardium. No labeling was detected above cardiomyocytes. Combined in situ hybridization and immunohistochemistry showed that the collagen mRNA producing cells have a myofibroblast-like phenotype in the infarcted myocardium and are fibroblasts in the non-infarcted septum and right ventricle. The increase in types I and III procollagen mRNA in both infarcted and non-infarcted myocardium was followed by an increased collagen deposition, measured by computerized morphometry on sirius red-stained tissue sections as well as by the hydroxyproline assay. In the non-infarcted septum and right ventricle the collagen-positive area was maximal at day 14 (3- to 5-fold increase compared with sham operated controls) and slightly declined at day 21. In the infarcted myocardium the collagen-positive area was 57 ± 10% at day 14 after MI. Hydroxyproline contents were significantly increased in the noninfarcted septum. A 2.3-fold increase was found 14 days after MI, while in the infarcted areas 3- and 8.2-fold increases were found 7 and 14 days after MI, respectively. We conclude that types I and III procollagen mRNA and protein content increase in both the infarcted and non-infarcted parts of the myocardium after MI. Interstitial cells and not cardiomyocytes produce these collagens, myofibroblasts in the infarcted and fibroblasts in the non-infarcted myocardium. (Am J Pathol 1995, 147: 325–338)

The fibrillar collagens, type I and type III, are the major components of the cardiac extracellular matrix.[1] The amount and distribution of these fibrillar collagens in the myocardium are an important denominator of cardiac function, and changes in the amount and/or distribution of collagen can affect the function of the heart.[2] For instance, an increase in intercellular and pericellular collagen fibers may limit myocyte motion and decrease the compliance of the ventricle.[3] Also,

Supported by grant NHS 90.282 from the Netherlands Heart Foundation.

Accepted for publication April 19, 1995.

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arrhythmias may result from increased collagen deposition by limiting the speed of diffusion of the depolarizing wave throughout the myocardium.[4] Fibrillar collagens accumulate in the cardiac interstitium during aging,[5] but also in a number of pathological conditions including cardiac hypertrophy induced by pressure overload. The increased cardiac collagen accumulation in this latter condition has been described in a number of species and is associated with an altered cardiac function.[3,6] The molecular mechanisms of collagen accumulation during cardiac overload are poorly understood. Procollagen genes are expressed throughout adult life in the heart, and the proteins are continuously synthesized and degraded.[7] Collagen accumulation is therefore the result of a shift in the balance of collagen synthesis and degradation. For instance, banding of the rat thoracic aorta not only increases the types I and III collagen mRNA contents in the heart, but also decreases collagen degradation. The net result is an increased collagen deposition.[8–10] Other forms of hypertrophy, such as thyroid hormone-induced cardiac hypertrophy, are associated with decreased cardiac collagen gene expression.[11]

The mRNAs of collagen types I and III were solely found in cardiac fibroblasts, suggesting that the interstitial collagen is synthesized in cardiac fibroblasts and not in cardiomyocytes.[12] Even less is known about changes in cardiac collagen gene expression after myocardial infarction (MI) and most studies are limited to changes in the infarcted area. There, the collagenous network is first degraded, which may lead to wall thinning and dilatation.[13,14] Subsequently, new collagen is synthesized and a scar is formed that prevents further dilatation and rupture of the infarcted wall. In the noninfarcted, hypertrophied areas accumulation of collagen protein has been described both in rats and humans,[15–17] and this increase is associated with disturbances in the conducting system and increased myocardial stiffness.[18] No data are available that relate changes in cardiac procollagen gene expression and collagen protein deposition in the hypertrophied non-infarcted myocardium, and localize and phenotype the cells involved in the production of collagen mRNA and deposition of collagen fibrils.

In the present study we have examined the time course of the mRNA contents of the types I and III collagen gene and the collagen protein content in the rat heart after induction of a myocardial infarction. Types I and III collagen mRNA and protein were localized by *in situ* hybridization and immunohistochemistry, respectively. Furthermore, types I and III collagen mRNA-producing cells were characterized with combined *in situ* hybridization and immunohistochemistry. The hypothesis was that both collagen mRNA and protein are increased in the infarcted and noninfarcted regions of the rat heart after MI and that collagen mRNA and protein are present within cardiac (myo-)fibroblasts and not in cardiomyocytes.

Materials and Methods

Animals

Adult male Wistar rats (Winkelmann, Bochum, Germany) were used, weighing 250 to 300 g at the start of the experiments. The experiments were performed according to the institutional guidelines for care and use of laboratory animals. All rats were given standard rat chow and water *ad libitum*.

Surgery

The animals were anesthetized with pentobarbital (60 mg/kg i.p.) and ventilated by positive pressure through an endotracheal tube attached to a Harvard small animal respirator (Edenbridge, UK). Via a left-sided thoracotomy and opening of the pericardium, the left descending coronary artery (LAD) was ligated, which results in infarction of the free left ventricular wall. After closing the chest, lidocaine (Xylocaïne, ASTRA, Rijswijk, The Netherlands) was given i.m. (2 mg/kg) to reduce the incidence of ventricular arrhythmias. The sham procedure consisted of a superficial suture in the epicardium of the left ventricle. After surgery the rats received food and water *ad libitum*. In total 209 rats were operated, 130 animals underwent coronary artery ligation and 79 rats sham operation. Overall survival rate was 63%. Furthermore, animals with an infarct size of less than 20% were excluded from the study, since previous studies have shown that in these animals no hemodynamic changes occur.[19] This resulted in 72 infarcted and 79 sham operated animals.

Northern Blotting

21 Infarcted and 21 sham operated rats (n = 3 per time point) were killed by ether 1, 2, 4, 7, 14, 21, and 90 days after surgery. The heart was quickly removed and a 1 to 1.5 mm coronal section, taken from the equator of the heart, was fixed in 10% formalin and embedded in paraffin for determination of infarct size. The rest of the tissue was divided in right ventricle, septum, and infarcted (MI) or non-infarcted (sham) left ventricular wall. The samples were rapidly frozen in liquid nitrogen and stored at −80°C. Total RNA was

isolated from the samples using the LiCl/urea extraction method.[20] 10 μg total RNA was dissolved in 30 μl sample buffer containing 10 mmol/L sodium phosphate (pH 7), DMSO, and glyoxal. After denaturation (1 hour at 50°C) the samples were electrophoresed in a 1% agarose gel with a 10 mmol/L sodium phosphate buffer for 3 hours at 100 V and subsequently transferred to a Hybond N^+ (Amersham, Little Chalfont, UK) membrane and immobilized by UV irradiation. Before hybridization the blots were stained with bromophenol blue, which stains the 18S and 28S ribosomal RNA bands. The intensity of the 18S ribosomal band measured by the Ultroscan XL Enhanced Laser Densitometer (LKB, Bromma, Sweden) was used as a marker for the amount of RNA loaded. Hybridization of RNA-cDNA was carried out overnight at 42°C with random primed ^{32}P-dCTP labeled rat α_1 type I procollagen cDNA (α1R1; *Pst*I digest: 1300 bp insert[21]), rat α_1 type III procollagen cDNA (RGR-5; *Eco*RI digest; 1300 bp insert[22] or glyceraldehyde 3-phosphate dehydrogenase (pGAPDH; *Eco*RI-*Bam*HI digest, 1007 bp) as a housekeeping gene, using standard conditions. Each blot was hybridized in random order with these three cDNAs. In between the different hybridizations the blots were boiled for 10 minutes in distilled water to remove the hybridized probe. The filters were exposed for 1 to 3 days (and for GAPDH, 2 to 3 hours) at room temperature to phosphor screens, and activity was measured by the Phosphor Imager (model 400) (Molecular Dynamics, Sunnyvale, CA) using the Imagequant software (Molecular Dynamics). The ratio of the band intensity of the procollagen types I or III mRNA band and the GAPDH band of that same sample was taken as the procollagen types I or III mRNA level in the sample. Ratio of the band intensity of procollagen types I and III mRNA and loaded RNA (18S band intensity) were also determined.

Infarct Size Measurement

Paraffin sections (4 μm) were stained with Azan, and infarct size was measured as a percentage of the circumference of the left ventricle[23,24] using the Quantimet 570 morphometer (Cambridge Instruments Ltd., Cambridge, UK). Mean infarct size was 41 ± 15% (mean ± SEM, n = 72).

In Situ *Hybridization*

For *in situ* hybridization a second series of animals was used. Infarct and sham animals (n = 5 per time point) were killed by ether 7, 14, 21, 35, and 90 days after surgery. The hearts were perfusion-fixed via a catheter in the abdominal aorta with 2% phosphate-buffered formaldehyde at a pressure of 100 mm Hg. After excision and overnight fixation in 4% phosphate-buffered formaldehyde, the heart was cut into four 2-mm slices from apex to base, dehydrated, and embedded in paraffin.

Paraffin sections (4 μm) were mounted on RNAse-free Vectabond-treated slides (Vector Laboratories, Burlingame, CA). The sections were deparaffinized and digested with 0.05% pepsin (Boehringer, Mannhein, Germany) in 0.2 N HCl for 30 minutes at room temperature. After a phosphate-buffered saline (PBS) wash, the sections were hybridized at 55°C with the above-mentioned, now ^{35}S-dCTP labeled collagen types I and III cDNAs.[25] After several washes the sections were dehydrated in graded series of ethanol, air dried, coated with Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), and exposed for various periods of time. The exposed slides were developed in Kodak D-19 developer, fixed, and counterstained with hematoxylin, dehydrated, and mounted. Serial sections were hybridized with a ^{35}S-dCTP-labeled empty plasmid as a negative control.

Collagen Positive Tissue Area

Paraffin sections (6 μm) of all four levels of each rat heart from the same series of rats as used for *in situ* hybridization were stained for 90 minutes with Sirius red (Polysciences, Warrington, PA).[26] The Sirius red stain has a high affinity for collagen and can directly be correlated with hydroxyproline contents of the tissue, a biochemical method for collagen quantification.[27–29] The collagen-positive area of the non-infarcted right ventricle, septum, and infarcted left ventricle were determined by morphometry (Quantimet 570 Image analyzer, Leica, Cambridge, UK).[16,30] One section of each level was measured. In each section 15 fields were analyzed (magnification ×25) in both the central part of the septum and the right ventricle (no peri- and endocardial fibrosis), omitting perivascular fibrosis. The investigator was blinded for the experimental group. Collagen-positive area was calculated as the area occupied by Sirius red-stained collagen divided by the total tissue area within the same microscopical view and expressed as the mean ± SEM of all fields measured within one heart.

Hydroxyproline Assay

A separate group of rats were used for the hydroxyproline assay. The rats were killed directly after

surgery or 7 and 14 days after sham or MI operation (n = 6 or 7 per time point). A central equatorial piece of cardiac tissue was excised, fixed in formalin, and used for infarct size measurement. The rest of the tissue was divided into left and right ventricle and septum, frozen in liquid nitrogen and stored at −80°C. About 100 mg wet weight septum and left ventricle was freeze dried and used for the hydroxyproline assay. The tissue was homogenized (Polytron, PT1200 tissue homogenizer, Kinematica AG, Switzerland) in 0.1 mol/L NaCl, 5 mmol/L $NaHCO_3$, washed and centrifuged five times with the same solution, for removal of proteins,[31] and hydrolyzed in 1 ml 6 N HCl. Hydrolysis was carried out for 18 hours at 105°C. The samples were filtered and vacuum dried. The residue was dissolved in 400 µl water, and the pH was raised to 6. Hydroxyproline content was determined from duplicate samples of 150 µl by the method of Chiariello et al[32] using a calibration curve of 0.5 to 5 µg 1-hydroxyproline. The data are expressed as µg collagen per mg dry weight, assuming that interstitial collagens contain an average of 13.4% hydroxyproline.[32]

Immunohistochemistry

Serial 5-µm frozen sections of a fourth series of rat hearts of animals with MI or sham operation killed at 7, 14, and 90 days after operation (n = 4) were used for a double staining, which included a Sirius red staining and collagen types I or III immunohistochemistry. The sections were stained with rabbit anti-human type I collagen (Chemicon, Temecula, CA) (1:200 in PBS + 1% bovine serum albumin (BSA)) or rabbit anti-rat type III collagen (Chemicon) (1:50 in PBS + 1% BSA) for 1 hour at room temperature, washed 3 times 10 minutes with PBS, and incubated 1 hour with fluorescein conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark). The sections were washed three times 10 min with PBS and visualized under a Leitz Dialux 20 fluorescence microscope (Leitz, Wetzlar, Germany). Normal rabbit serum used in the same dilution as the primary antibodies and sections without primary antibodies were used as negative controls (data not shown).

Combined in Situ *Hybridization and Immunohistochemistry*

For combined *in situ* hybridization and immunohistochemistry, paraffin sections were treated and incubated similarly to the ones used for normal *in situ* hybridization with the exception that after deparaffinization the sections were treated with methanol + 0.3 % H_2O_2 for 20 minutes at room temperature. After hybridization and washing as described above, the sections were incubated three times 5 minutes with PBS and subsequently incubated with primary antibodies or lectin (see below) for 60 minutes at room temperature. The probes used were 1) rabbit anti-desmin (Eurodiagnostica, Apeldoorn, The Netherlands), 2) rabbit anti-vimentin (Eurodiagnostica), 3) mouse anti-α-smooth muscle actin (Dako), and 4) biotin-labeled lectin *Griffonia simplicifolia* (Sigma Chemical Co., St. Louis, MO). After incubation the sections were washed three times 5 minutes at room temperature and incubated for 30 minutes with PBS at room temperature with biotin-labeled goat anti-rabbit IgG (Dako) for the polyclonal antibodies, or biotin-labeled rabbit anti-mouse IgG (Dako) for the monoclonal antibody. After washing three times 5 minutes with PBS, all sections were treated with StreptABComplexes (Dako) for 30 minutes at room temperature, and washed three times 5 minutes with PBS. Peroxidase activity was visualized by incubation with diaminobenzidine. The sections were incubated with increasing concentrations of ethanol, air dried overnight, and dipped in Kodak NTB2 nuclear emulsion, incubated, developed, and counterstained as described above.

Statistics

All grouped data are expressed as mean ± SEM and compared by two-way analysis of variance.[33] The level of significance was taken at $P < 0.05$.

Results

Northern Blotting

Sham Operated Rats

Both types I and III procollagen mRNA could easily be detected in the left and right ventricles of both sham and infarct animals. In the sham animals low procollagen types I and III mRNA levels were detected in all three areas measured (free left ventricular wall, septum, and right ventricle), and the values remained low at all time points measured. A small transient increase in collagen type I mRNA level was noted 7 days after surgery in the left ventricle (Figures 1–3).

Infarcted Rats

Infarcted Area. In the infarct area significant increases of both types I and III procollagen mRNA levels were observed as compared with the values ob-

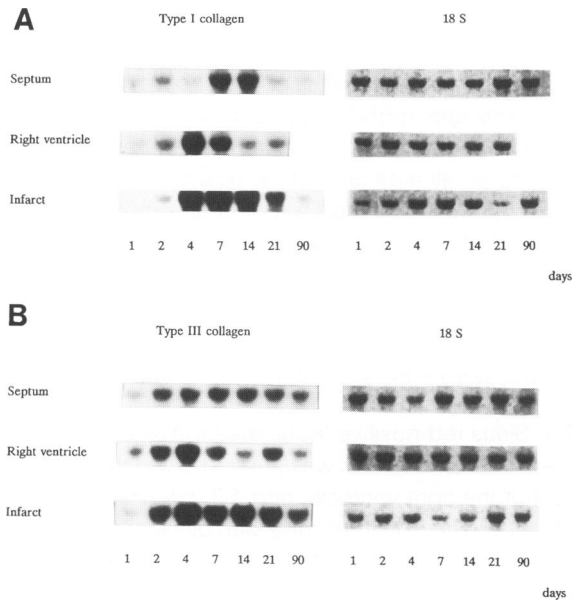

Figure 1. *Autoradiograms of Northern hybridization analysis of total RNA from the noninfarcted septum and right ventricle and the infarcted left ventricle 1, 2, 4, 7, 14, 21, and 90 days after operation and hybridized with specific cDNA probes for rat α1(I) procollagen* (A) *and rat α1(III) procollagen* (B) *using the bromophenol blue stained 18S band as a control.*

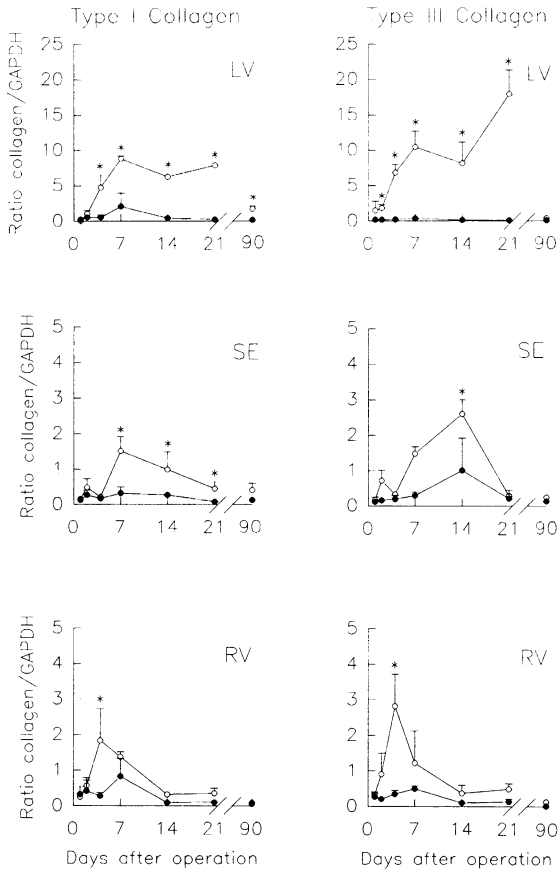

Figure 2. *Results of the densitometric scanning (Types I and III procollagen versus GAPDH) of the autoradiograms after Northern blotting of left ventricle (LV), interventricular septum (SE) and right ventricle (RV).* (○) *Infarcted animals.* (●) *Sham operated animals.* ($n = 3$ *to 4, mean ± SEM,* $^*P < 0.05$).

tained in the free left ventricular wall of sham animals using GAPDH as a control probe. Type III procollagen mRNA levels significantly increased as soon as 2 days after infarct induction (4-fold increase). Type III procollagen mRNA levels increased from day 2 to day 14 after infarction (11- to 14-fold increase), reached a peak at day 21 (30-fold increase), but returned to almost sham level at day 90 after infarct induction (Figures 1 and 2). Type I procollagen mRNA levels increased later than type III procollagen mRNA. A significant increase in type I procollagen mRNA levels was observed from day 4 (10-fold increase compared with the sham operated rats) and showed a plateau from day 7 to day 21 (10- to 15-fold increase). Procollagen type I mRNA levels remained increased, even 90 days after infarct induction (7-fold increase) (Figures 1 and 2).

Septum. Induction of MI also increased the mRNA levels of type I procollagen mRNA in the non-infarcted septum (Figures 1 and 2) as compared with the levels obtained in sham animals. The increases were smaller than in the infarcted area. In the septum type I procollagen mRNA levels were elevated from 7 to 21 days after infarct induction (4 to 5-fold increase compared with sham operated animals) and declined thereafter. Comparable data were obtained for type III procollagen (Figures 1 and 2). Type III procollagen mRNA was significantly increased at 7 days after operation in the septum of infarct animals (4 to 5-fold increase) as compared with the values obtained in the septum of sham animals. Type III procollagen mRNA levels declined to sham level at day 14 after infarction.

Right Ventricle. A significant increase in both types I and III procollagen mRNA levels could only be observed in right ventricle at 4 days after MI (5- and 7-fold, respectively, compared with sham operated controls). Using the 18 S ribosomal band intensity for normalization of total loaded RNA a 3-fold higher type III collagen mRNA transcription was noticed compared with type I mRNA from 4 to 21 days after MI in the infarcted left ventricle. Type III collagen mRNA levels were still elevated 90 days after MI in infarcted but also non-infarcted areas of the myocardium. A transient increase in type I collagen mRNA production was noticed in septum from day 2 to 14 and for type III collagen from day 2 to 90. In right ventricle type I collagen mRNA levels were increased from day 4 to 21 after operation and type III collagen from day 2 to day 90 (Figure 3).

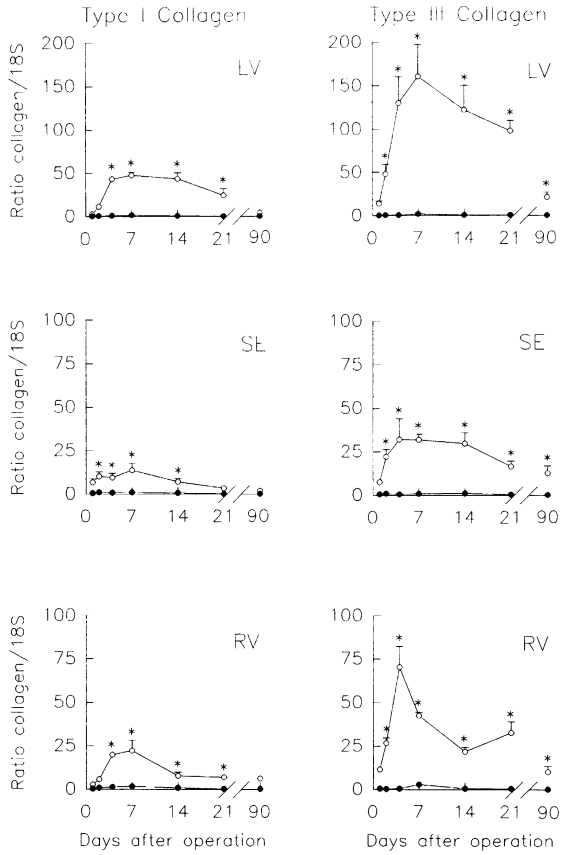

Figure 3. *Results of the densitometric scanning (Types I and III procollagen* versus *18S) of the autoradiograms and bromophenol blue-stained Northern blots of left ventricle (LV), interventricular septum (SE), and right ventricle (RV). (○) Infarcted animal. (●) Sham operated animals. (n = 3 to 4, mean ± SEM, *P < 0.05).*

In Situ *Hybridization*

Positive hybridization signals with procollagen type I and III mRNAs were found surrounding the necrotic area (Figure 4, A and B) in the infarcted part of the left ventricle at 7 days post-infarction. Grains were localized above small interstitial cells in the direct vicinity of the necrotic cardiomyocytes. No hybridization could be detected above cardiomyocytes. Using parallel tissue sections no differences in localization between types I and III collagen mRNA synthesizing cells could be observed (data not shown).

In the non-infarcted septum and the right ventricle of infarcted rats, hybridization of both types I and III procollagen mRNA was found above interstitial cells in between cardiomyocytes but never above cardiomyocytes (Figure 4, C and D). Furthermore, increased hybridization signals of types I and III procollagen mRNA were observed in the pericardium and in the adventitia of coronary arteries (data not shown). Compared with the infarcted left ventricle far less type I and III procollagen mRNA is synthesized in the non-infarcted myocardium because fewer cells are involved in collagen mRNA production (Figure 4, B and D). In sham animals no increase in types I and III procollagen mRNA hybridization signals were noticed (Figure 5, A and B) except for the epicardium and the small area around the suture in the left ventricle (Figure 5, C and D). Using an empty labeled plasmid vector as a negative control did not show any hybridization in sham and infarcted animals (data not shown).

Collagen-Positive Area

The Sirius red-positive tissue area (collagen-positive area) in sham animals was 2.4 ± 0.3% in the central part of the right ventricle, and 1.7 ± 0.4% (mean ± SEM, n = 7 to 8) in the central part of the septum. The collagen fractions of both the non-infarcted septum and right ventricle were already increased on day 7 after MI and maximal on day 14 in both septum and right ventricle (Figure 6). At day 14 after MI a 3-fold increase was observed in the right ventricle, whereas a 5-fold increase was found in the septum in comparison with sham operated animals at day 14. After day 14 the collagen-positive area showed an insignificant tendency to decline. In the infarcted left ventricle the collagen-positive area fraction was 57 ± 10% (mean ± SEM, n = 7 to 8) at day 14 after MI. In this measurement the area of necrosis was included in the total tissue content.

Hydroxyproline Assay

The collagen contents of septum and left ventricle were also determined by the hydroxyproline assay. The non-infarcted septum showed a significant increase (2.2-fold compared with sham control) in collagen content not earlier than 14 days after MI (Figure 7A). The collagen content in the infarcted left ventricle was already increased at 7 days after MI (3.1-fold increase) and increased even further at 14 days (8.2-fold increase compared with sham operated controls) (Figure 7B).

Immunohistochemistry

Parallel paraffin sections were stained with polyclonal antibodies to types I or III collagen (figure 8, A and B, respectively) and with Sirius red (Figure 8C). The edge of the infarcted left ventricle 14 days after MI showed a massive increase in collagen protein deposition shown with Sirius red staining. The same collagen fibers were stained in parallel sections with antibodies to types I or III collagen. At days 7, 14, and

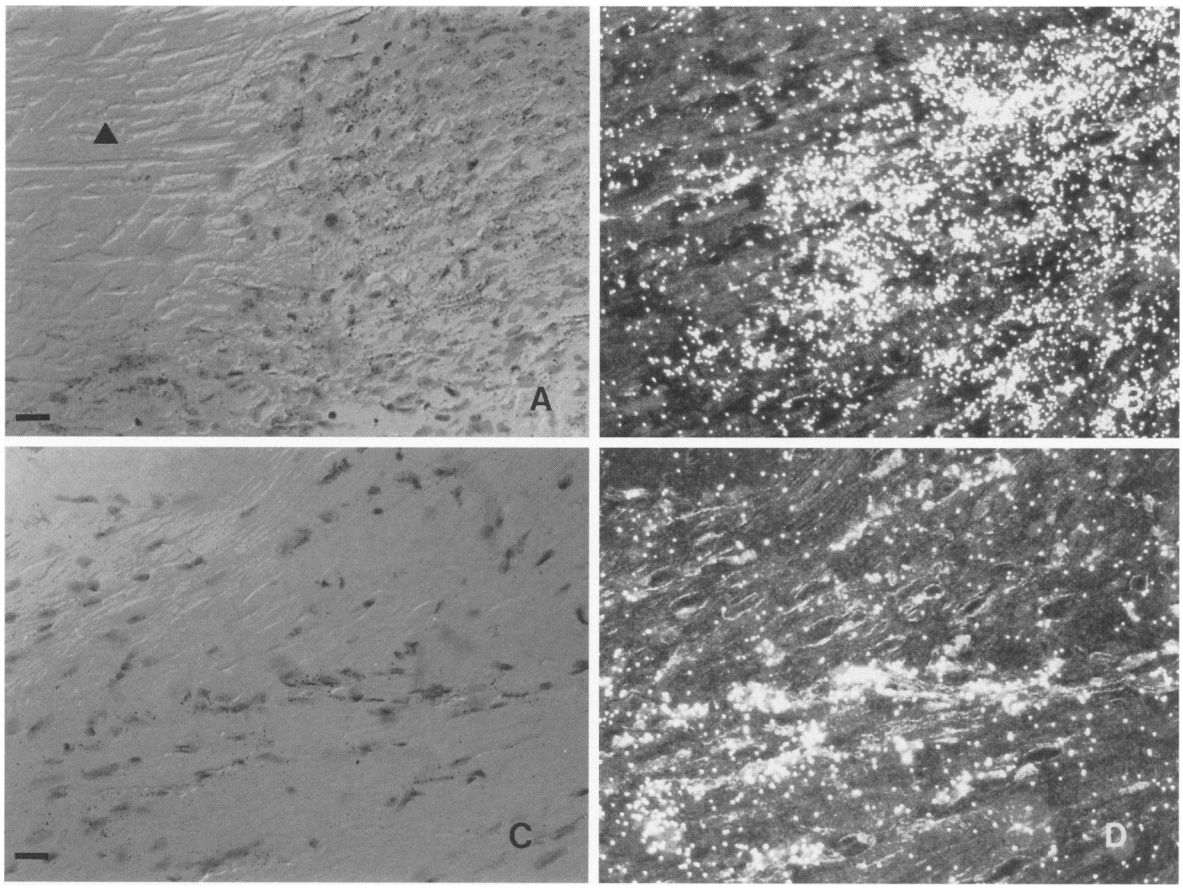

Figure 4. In situ *hybridization of* $\alpha_1(I)$ *procollagen mRNA in the infarcted rat heart 7 days after MI. In the infarcted left ventricle abundant* $\alpha_1(I)$ *mRNA synthesizing cells were observed surrounding the area of necrosis* (▲) (**A** *and* **B**). *Localization of* $\alpha_1(I)$ *mRNA exclusively between cardiomyocytes in the non-infarcted septum after MI* (**C** *and* **D**). (**A** *and* **C**) *Light microscopical view.* (**B** *and* **D**) *Dark field illumination of* **A** *and* **C**, *respectively. Bars = 20 μm.*

21 after MI increased collagen deposition could be found surrounding the necrotic cardiomyocytes in the left ventricle, as well as an increased perivascular and interstitial collagen deposition in the noninfarcted septum and right ventricle and in the pericardium due to opening of the pericardium during operation. The increased collagen deposition in the pericardium of infarcted animals was not different from that observed in sham operated animals. Colocalization of types I and III collagen fibers could be observed in all places of collagen deposition. Even at 90 days after MI, when the area of necrosis is completely remodeled and occupied by extracellular matrix components, capillaries, fibroblasts and α-smooth muscle actin containing elongated mesenchymal cells (see below), both type I and type III collagen could be observed. Not only in the remodeled left ventricle but also in the noninfarcted myocardium in between cardiomyocytes and in the adventitia of coronary arteries colocalization of types I and III collagen could be demonstrated (data not shown).

Characterization of Types I and III Procollagen mRNA-Synthesizing Cells

Type I collagen mRNA-producing cells were furthermore characterized by combined *in situ* hybridization and immunohistochemistry. Hybridization of tissue sections 7 days after MI with the type I collagen probe followed by staining with biotin-labeled *Griffonia simplicifolia*, a lectin that specifically binds to endothelial cells, showed that in the infarcted (Figure 9, A and B) and noninfarcted myocardium the type I collagen mRNA-producing cells never stained with this lectin. This indicates that the collagen mRNA-producing cells are not endothelial cells. Parallel sections hybridized with the type I collagen probe and stained with an antibody to vimentin showed that almost all collagen mRNA-producing cells stained with the vimentin antibody (Figure 9, C and D), suggesting that the collagen mRNA-producing cells are of mesenchymal origin. The collagen mRNA-producing cells never stained with a polyclonal antibody to desmin, which

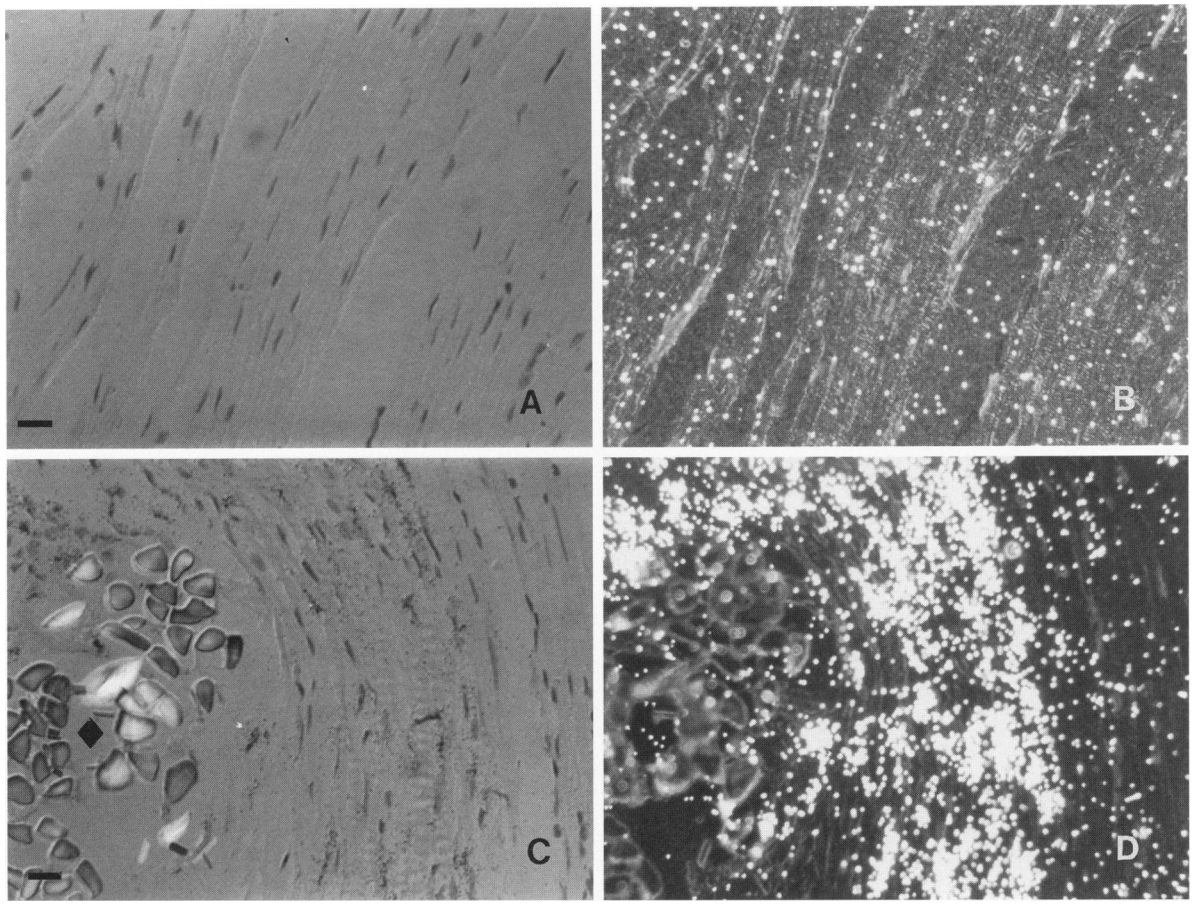

Figure 5. In situ *hybridization of α_1(I) procollagen mRNA in sham operated rat heart 7 days after operation. In the sham operated left and right ventricle and septum no hybridization signal was observed.* **A** *and* **B** *show an example of the septum of a sham operated rat. In sham operated rats α_1(I) procollagen mRNA expression could only be detected surrounding the superficial suture (♦) (*C *and* D*). (*A *and* C*) Light microscopical view. (*B *and* D*) dark field illumination of* A *and* C*, respectively. Bars = 20 μm.*

indicates that these cells are not myocytes (Figure 9, E and F). In the infarcted left ventricle 7 days after MI around the area of necrosis almost all collagen mRNA-producing cells showed expression of α-smooth muscle actin (Figure 9, G and H), which suggest that the collagen is produced by an activated fibroblast or myofibroblast-like cell. In the non-infarcted septum or right ventricle, however, the type I collagen mRNA-producing cells showed immunostaining with vimentin but not with α-smooth muscle actin. Similar experiments using the type III collagen probe showed the same results (data not shown). The empty labeled plasmid vector did not yield any hybridization in sham and infarcted animals (negative control, data not shown).

Discussion

In the infarcted area, mRNAs of both types of procollagen showed different expression patterns over the time period measured. Type III procollagen mRNA preceded the increase in type I procollagen mRNA. This pattern resembles the expression of types I and III collagen described in other forms of wound repair, eg, dermal wound healing.[34] Early in dermal wound healing a network of type III collagen and other extracellular matrix components such as embryonic fibronectin isoforms is formed, which serves as a scaffold for the deposition of the resilient type I collagen fibrils. Also in the heart, embryonic fibronectin isoforms are re-expressed early (day 1 to 2) after MI.[35,36] In contrast to the transient collagen mRNA expression in dermal wound healing, collagen mRNA synthesis is still elevated 3 months after infarction in the infarcted left ventricle. The sustained increase in collagen mRNA in the infarct may be explained by the unique environment of the wound in the heart, which undergoes continuous cycles of stretching and relaxation. Cyclic stretching induces increased collagen protein synthesis in cultured smooth muscle cells and urothelium.[37] Infarct and dermal wound healing differ in more aspects. For instance, myofibroblasts,

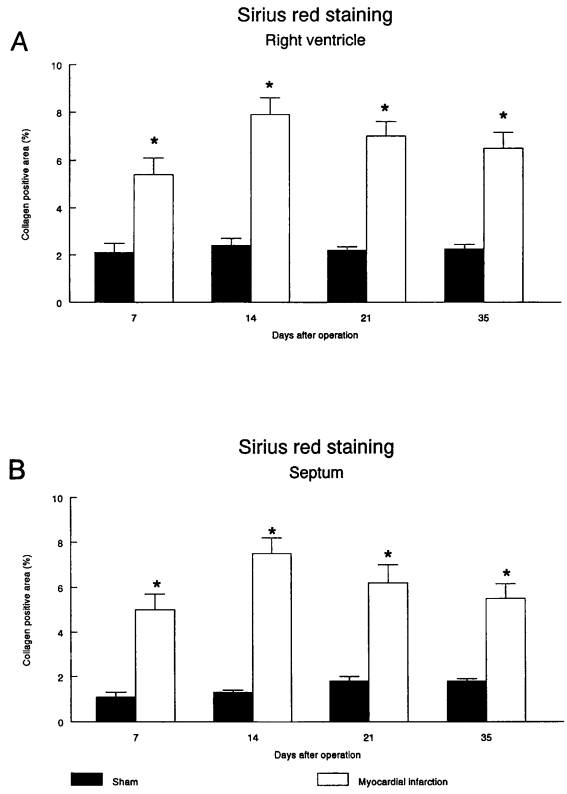

Figure 6. *Sirius red-positive area of sham operated and infarcted right ventricle* (**A**) *and septum* (**B**). (*n = 7 to 8, mean ± SEM,* *P < *0.05.*)

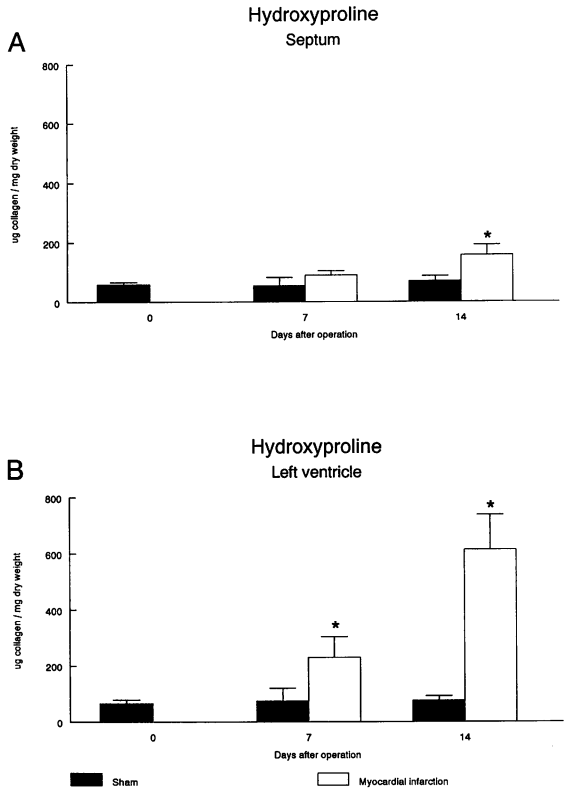

Figure 7. *Results of the hydroxyproline assay of interventricular septum* (**A**) *and left ventricle* (**B**) *of sham and infarcted rats: 0, 7, and 14 days after operation.* (*n = 6 to 7, mean ± SEM,* *P < *0.05*).

which play a role in wound contraction, are only transiently present in a dermal wound,[38] but maintain a presence in rat and human infarcts for prolonged time periods.[39,40] As demonstrated in this study by the use of combined *in situ* hybridization and immunohistochemistry, the type I and III collagen mRNA-producing cells surrounding the area of necrosis in the left ventricle are mesenchymal cells (vimentin-positive), not endothelial (*G. simplicifolia* lectin-negative) nor muscle cells (desmin-negative) but contain α-smooth muscle actin, suggesting that these cells have a myofibroblast-like phenotype.[41] The observations that myofibroblasts most likely originate from fibroblasts and that the collagens are derived from these activated fibroblasts, as observed in this study and by others,[8,12] may explain the parallel expression patterns of the collagen mRNAs and the myofibroblastic phenotype in dermal and myocardial wound healing.

The increases in procollagen mRNAs in the non-infarcted septum and right ventricle were different from the above discussed changes in the infarct. They were lower and present in a narrower time span, but do resemble the changes in procollagen mRNA in rat cardiac pressure overload hypertrophy.[9,42] After aortic coarctation, types I and III procollagen mRNA levels are also transiently increased in the first week. The increased collagen mRNA levels after MI at the earlier time points (day 1–7) could, however, be due to an overall increase of mRNA transcription during the development of compensatory hypertrophy. GAPDH used as the reference gene control for this nonselective increase in mRNA transcription showed only a slight insignificant increase *versus* sham operated rats during the first days after MI, which indicates that infarction induces a selective increase in types I and III collagen mRNA in the infarcted and noninfarcted myocardium. In humans with ischemic cardiomyopathy Mukherjee and Sen[43] found that the increased collagen concentration was only due to increased type III collagen production, whereas Bishop et al[44] showed a higher type I collagen deposition in myocardial diseases. Although we did not determine the collagen subtypes biochemically, our Northern blot and *in situ* hybridization results demonstrate that both type I and III collagen mRNA levels are increased. The cells producing types I and III collagen mRNA in the non-infarcted myocardium were vimentin-positive and α-smooth muscle-negative. Given their localization and elongated shape, these

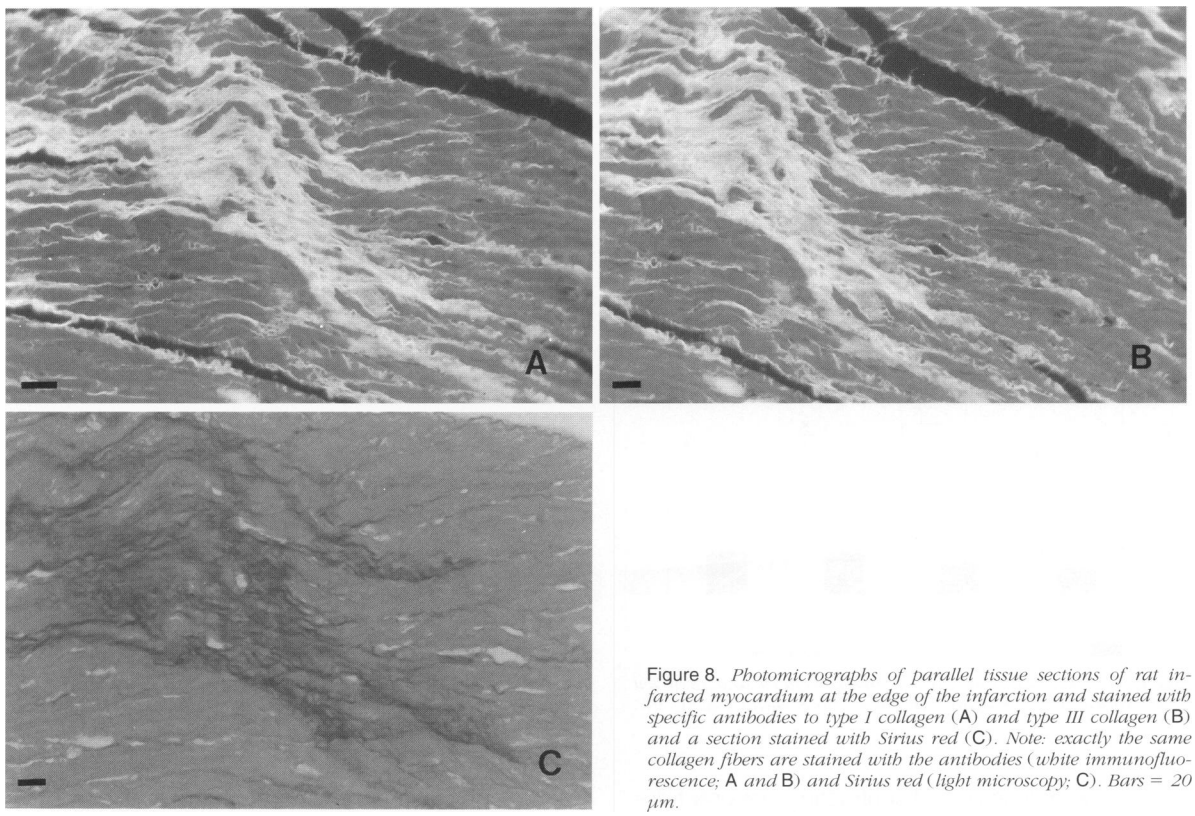

Figure 8. *Photomicrographs of parallel tissue sections of rat infarcted myocardium at the edge of the infarction and stained with specific antibodies to type I collagen* (A) *and type III collagen* (B) *and a section stained with Sirius red* (C). *Note: exactly the same collagen fibers are stained with the antibodies (white immunofluorescence;* A *and* B) *and Sirius red (light microscopy;* C). *Bars = 20 μm.*

cells look like normal fibroblasts and do not show a shift toward a myofibroblastic phenotype as occurs during wound healing in the skin and in the area of infarction surrounding the area of necrosis.

The increase in procollagen mRNA was associated with an increase in collagen protein deposition in both the infarcted and non-infarcted areas. The observation that the time sequences of mRNA expression and protein deposition of types I and III collagen were attuned to each other suggests a transcriptional control of collagen protein production. In the infarct the new collagen fibers surrounded the necrotic parts from day 7 after MI on and in time necrotic cardiomyocytes were replaced by interstitial collagen and other extracellular matrix components. This extracellular meshwork, the so-called replacement fibrosis[1] will give the infarct more resilience and tensile strength and can prevent rupture and dilatation. At day 14 after MI the collagen-positive area fraction was 57 ± 10%, which is a 30- to 40-fold increase compared with sham operated animals.

In both infarcted and non-infarcted myocardium a significant increase was observed in the hydroxyproline content (collagen content). These data support the findings that the collagen volume fraction increased not only in infarcted but also in non-infarcted myocardium. A good correlation between the collagen volume fraction determination and the collagen content by hydroxyproline has been described earlier.[27]

Besides synthesis, interstitial fibrillar collagen can be degraded by specific matrix metalloproteinases (MMP-1 and MMP-8).[45,46] In the infarcted left ventricle collagenolytic (MMP-1) activity is increased from day 2 till day 7 after MI. This increased collagenolytic activity is involved in the remodeling of the area of myocyte necrosis[47] rather than in the early degradation of collagen struts as observed within 1 day after ischemia or myocardial stunning.[48–50] In this rat infarction model the net result of collagen synthesis and degradation is increased collagen deposition in the noninfarcted myocardium and scar formation in the infarcted area.

Collagen protein increase (2- to 5-fold increase compared with the sham operated rats) in the non-infarcted areas was not related to myocyte necrosis

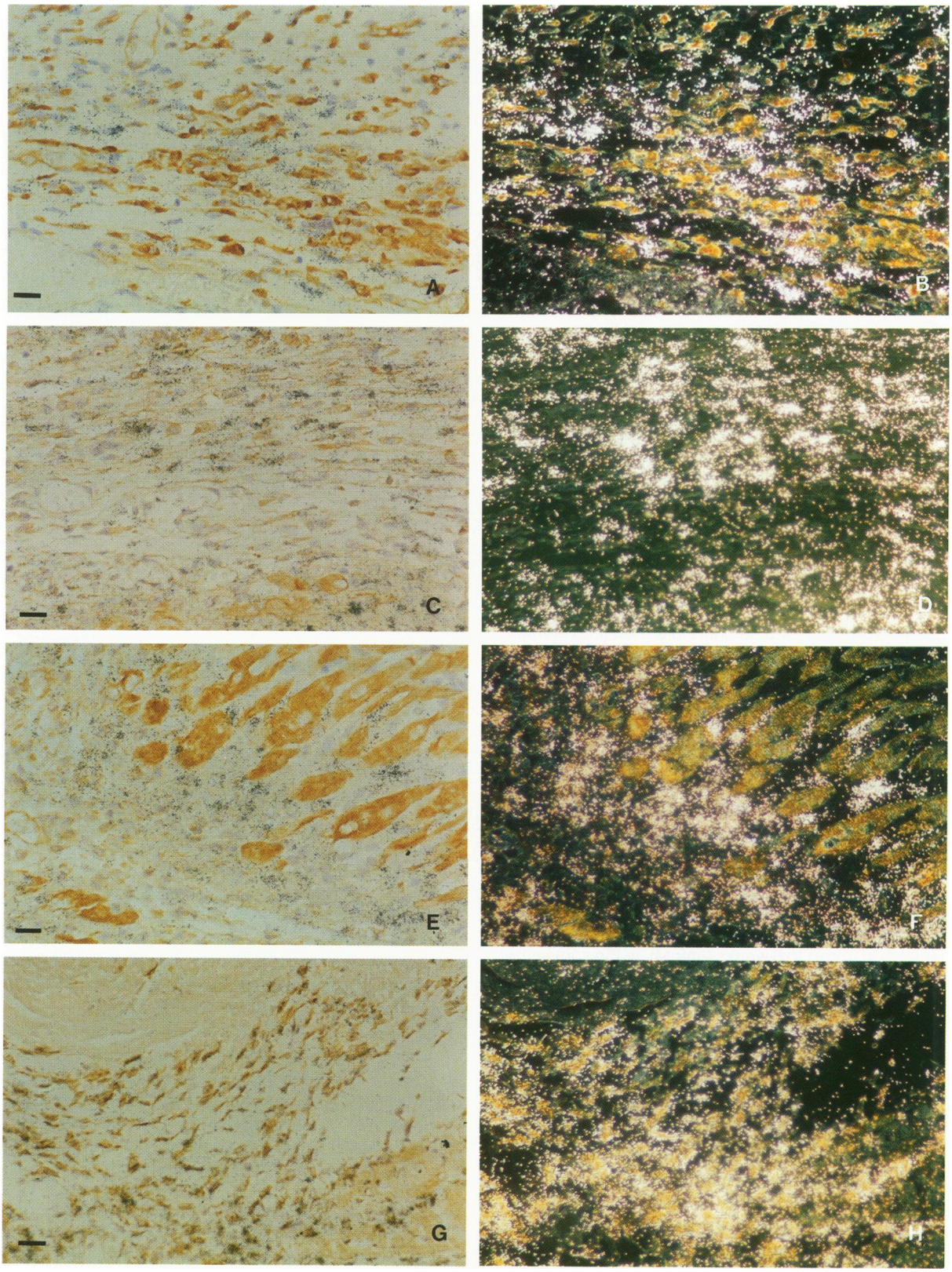

Figure 9. *Photomicrographs of tissue sections (6 days after MI) used for combined* in situ *hybridization with the type I collagen probe followed by immunohistochemistry with:* G. simplicifolia *lectin (A and B), vimentin (C and D), desmin (E and F), α-smooth muscle actin (G and H) for the infarcted LV. A, C, E, and G are bright field photomicrographs, and B, D, F, and H are their dark field illuminations. Bars = 20 μm.*

and resembled the pattern of reactive fibrosis as described in hypertension-induced hypertrophy.[51,52] This type of interstitial fibrosis is characterized by thickening of perimysial tendons, denser perimysial weaves, and occupation of intermuscular spaces by collagen fibers and increased perivascular fibrosis around most of the coronary arteries (not measured by the Sirius red experiment).[6] Also in humans increased collagen deposition was found in the non-infarcted interventricular septum after a myocardial infarction.[17] Although this study clearly demonstrates increased type I and III collagen mRNA and protein production in the infarcted and non-infarcted areas of the rat heart after MI, the molecular mechanisms controlling collagen deposition are still unclear. The results from this and other studies may suggest, however, some possible candidates. First, the data indicate that the regulation of collagen in the infarct may differ from that in the non-infarcted area. As discussed above, collagen deposition in the infarct is part of a wound healing response and may be further triggered by the cyclic stretching of the wound as shown by the occurrence of a myofibroblastic phenotype. In contrast, in the non-infarcted area, a circulating factor rather than a local factor has to be considered, given that the non-infarcted left ventricle and right ventricle show similar changes in collagen mRNA and protein levels. A long list of possible factors, including angiotensin II, aldosterone, and catecholamines,[53,54] are, at least *in vitro*, capable of affecting collagen metabolism. Recent data obtained in our and other laboratories indicate that the renin angiotensin aldosterone system is indeed involved in the regulation of collagen deposition, at least in the non-infarcted areas. Sun et al[55] demonstrated that angiotensin-converting enzyme binding is coincident with fibrillar collagen accumulation. In the heart after MI angiotensin-converting enzyme binding density increased from 1 to 8 weeks after MI. In the same time period increased collagen deposition is localized in the same area. Both angiotensin-converting enzyme inhibition and angiotensin II receptor subtype I blockade inhibit the increase in collagen deposition early after infarct induction.[16,56] Also, hemodynamic changes after MI can lead to neurohumoral activation and affect ventricular remodeling in both left and right ventricle. However, one cannot exclude that these effects are indirect, and that other factors, such as TGF-β, are involved.[57] TGF-β_1 plays a key role in embryogenesis and wound healing[58] and is a potent stimulator of type I and III collagen mRNA production and protein deposition.[59] TGF-β_1 mRNA levels are increased after myocardial ischemia[60] and pressure overload hypertrophy,[9] and this response precedes extracellular matrix mRNA and protein synthesis. TGF-β_1 also modulates the phenotype of fibroblasts into myofibroblasts.[41,61] These myofibroblasts are described as the most important connective tissue-producing cell type in the liver, and use TGF-β as the main fibrogenic mediator.[62]

In conclusion, the present study demonstrates that types I and III procollagen mRNA and protein are produced by non-cardiomyocytes, fibroblasts in the non-infarcted, and myofibroblasts in the infarcted myocardium. Types I and III procollagen mRNA and protein are increased in both the infarcted and the non-infarcted myocardium after MI in the rat. The results suggest 1) that the regulation of collagen synthesis in the infarct may be different from the regulation of collagen synthesis in the non-infarcted areas of the heart, and 2) that collagen synthesis in the infarcted cardiac tissue is regulated differently than collagen synthesis in a dermal wound, which shows a more transient collagen production and myofibroblastic phenotypic switch.

Acknowledgments

The authors wish to thank J. Debets for excellent technical assistance and Dr. D. Rowe and Dr. E. Vuorio for the gift of the types I and III procollagen cDNA probes.

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