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Left ventriculotomy of the heart: tissue repair and localization of collagen types I, II, III, IV, V, VI and fibronectin

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Summary. The reparative process following left ventriculotomy was investigated immunohistochemically using anti-type I, II, III, IV, V and VI collagen antibodies, and anti-fibronectin antibody. Wound healing began with proliferation of young fibroblasts positive for type I, III and V collagens at the wound margin; vascular granulation tissue then grew into the injured myocardium followed by deposition of fibrous components immunoreactive with type I and III. At 30 days after operation when almost the entire thickness of the myocardium at the wound was replaced by fibrosing granulation tissue, a small cluster of adipocytes appeared around capillaries at the wound margin. The granulation tissue was gradually replaced by the adipose tissue with establishment of a fibrous union at the subendocardium by 90 days. In addition to type I and III collagens, type VI collagen was detected in a fine fibrillary pattern along thick collagen fibre bundles in the fibrous tissue and around the adipocytes. Fibronectin was distributed diffusely in the granulation tissue and gradually decreased with increase of the fibrous components. These results indicate that the ventriculotomy was finally repaired in the form of a fibrous scar, particularly in the endocardium. There was marked infiltration of adipose tissue in the damaged myocardium. Presumably type VI collagen, as well as type I and type III collagens, plays an important role in wound union.

Key words: Heart – Wound repair – Collagen types – Dog

Introduction

Wound healing after the myocardial injury is influenced by specific factors. The heart beats and the wound is subjected to a high pressure load without immobilization; also, myocytes have a poor regenerative capacity. There have been a few reports (Carter and MacMillian 1950; Dillon and Postlethwait 1961; Thomas et al. 1952; Frankel et al. 1961) on this subject particularly concerning transmural ventriculotomy, although surgical left ventriculotomy has been performed successfully for the treatment of ventricular tachycardia of left ventricular origin and for left ventricular fibroma (Iwa et al. 1988). In 1950 Carter and MacMillian first described the sequence of the wound repair after left ventriculotomy that terminated in a fibrous scar. In their view this was because of the poor regenerative capacity of cardiac muscle. Recently, however, Iida (1983) reported that the damaged myocardium by a cryo-injury was eventually replaced by an adipose tissue from the epicardium.

In secondary union of a wound, collagen type I, III and fibronectin are major components of extracellular matrix (Kurkinen and Vaheri 1980; Williams et al. 1984) and play an important role in determing the tensile strength. It is known that minor collagens, type V (von der Mark et al. 1984; Gibson and Cleary 1983) and VI (Mayne 1986; Bartholomew and Anderson 1983) are widely co-distributed in the interstitial tissue with major collagens and that in the myocardium, type IV collagen is present as a scaffold for the myocytes (Martinez-Hernandez and Amenta 1983; Vracko et al. 1988).

The present study was performed to elucidate the sequence following left ventriculotomy and the dynamic changes of tissue localization of collagen types I, II, III, IV, V, VI and fibronectin in the entire course of the wound healing.

Materials and methods

Left ventriculotomy was performed on 25 adult mongrel dogs. Dogs were anaesthetized by intramuscular injection of 10 mg/kg ketamine-hydrochloride and by intravenous injection of 20 mg/kg pentobarbital. The operation was performed as follows; using an artificial ventilator of Harvard type; the heart was exposed at the level of fifth intercostal space after thoracotomy. A longitudinal and transmural incision 2.0 cm in length was made in a left ventricular apex and closed by mattress sutures with Teflon felts (Figs. 1,

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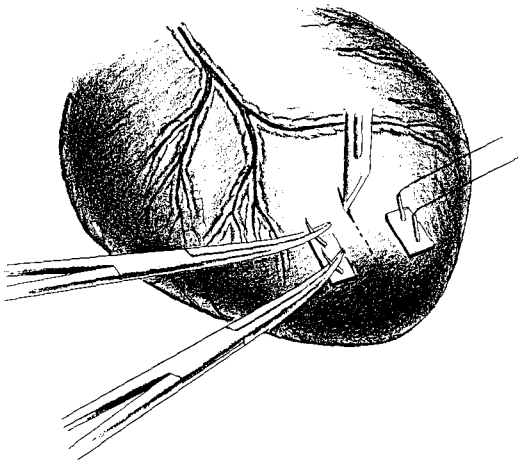

Fig. 1. A drawing showing the present left ventriculotomy

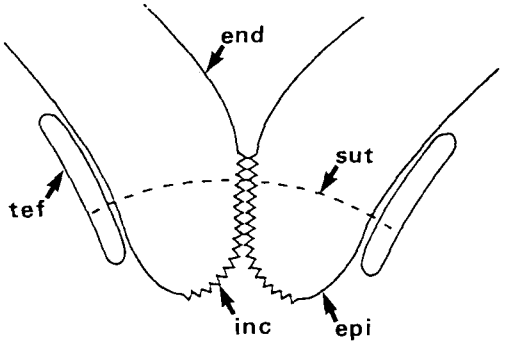

Fig. 2. A schematic representation of a cross-section of a transmural incision that is sutured tightly using Teflon mattress. *inc*, Incision; *sut*, suture; *tef*, Teflon; *end*, endocardium; *epi*, epicardium

2). Antibiotics were injected intramuscularly for 3 days after operation. Three animals were killed 1, 3, 6, 10, 20, 30, 45, and 90 days after the operation. The non-contractile area of the left ventricle in the heart was measured by a planimeter and the ratio of the non-contractile area to the total left ventricular area calculated. Half of the wound was fixed with neutral-buffered 10% formalin, and half was frozen in liquid nitrogen without fixation.

Haemodynamic studies were done for dogs which survived over 30 days. The blood pressures in the aortic arch, pulmonary artery, central vein and pulmonary vein, the pulmonary artery wedge pressure, and the cardiac output were monitored both before and after the operation. The same procedures were repeated after sham thoracotomy. Values for a haemodynamic variables between, before and after left ventriculotomy were compared, and Dunnett's multiple comparison method was used for the differences of the mean values. Less than 5% probability was evaluated as a significant difference.

Anti-human collagen type I, III and V polyclonal antibodies were raised in rats. Anti-bovine collagen type II polyclonal antibodies and anti-human collagen type VI antibodies were raised in rabbits. Type-specific polyclonal anti-collagen antibodies were purified by cross-adsorption of affinity columns consisting of type I, II, III, IV, V and VI collagen as previously described (Minamoto et al. 1988; Oda et al. 1988; Ueda and Nakanishi 1989). Anti-human collagen type IV monoclonal antibody was kindly provided by Dr. Funabiki. Anti-dog plasma fibronectin polyclonal antibody was raised in a rabbit and was affinity-purified as described previously (Kawahara et al. 1989).

Formalin-fixed and paraffin-embedded sections were immunostained by the avidin-biotin-peroxidase complex method. Sections were pre-treated by 0.05% protease (type XXIV, Sigma, USA) for 30 min at 37° C for type I, II, III, and V and 0.01% pepsin (Sigma, USA) for 2 h at 37° C for type IV collagen. Primary antibodies were incubated overnight at 4° C. The working dilutions of antibodies to collagen type I, II, III, IV, and V were 40, 20, 40, 10, and 20, respectively. Unfixed frozen sections were processed for type V and VI collagen, and fibronectin by an indirect immunofluorescence method. Primary antibodies were incubated for 2 h at room temperature and the working dilutions to type V and VI collagen and fibronectin were 20, 200 and 100, respectively. An immunofluorescence method was employed for type V and VI collagen and fibronectin in the present studies because immunoreactivities for type V collagen and fibronectin were much clearer and more reliable by immunofluorescence than immunoperoxidase. This was particularly true for intracytoplasmic positivity of type V collagen, which was demonstrable without the problems of activity of intrinsic peroxidase. As to type VI collagen, the anti-human collagen type VI antibody we prepared did not cross-react to dog tissues in the formalin-fixed state but worked with the unfixed frozen section. Other antibodies used in this experiment were checked by positive cross-reaction to the corresponding type of collagens between the dog and human kidney tissues.

Negative controls were stained simultaneously, using normal rat or rabbit IgG instead of anti-collagens or anti-fibronectin antibodies.

Results

All values for haemodynamics were within normal limits. The ratio of the non-contractile area in the left ventricle was $7.9 \pm 3.2\%$ (mean $\pm$ standard deviation), effectively small and negligible in the maintainance of cardiac function. Thus, the present operation was considered to be a safe procedure.

The histological findings in the wound in the exudation phase (1–3 days) included ischaemic necrosis of the entire thickness of the myocardium at the ventriculotomy, probably due to the tight mattress suture. The necrosis was characterized by eosinophilic myocytes with loss of cytoplasmic cross-bands and contraction bands in the boundary zone close to viable cells. A fibrin thrombus formed on the endocardial surface and an exudate of fibrin was seen on the pericardium and along incisional lines. At 3 days after operation neutrophil infiltration became dominant in necrotic areas. In the central necrotic portion, myocytes were liquefied with dense neutrophil infiltration. Oval fibroblasts with prominent nucleoli and plump cytoplasm proliferated in the marginal zone of necrosis (Fig. 3a) and migrated in the direction of the central necrosis. Proliferation of fibroblasts was prominent in the endocardium (Fig. 3b) and epicardium. The extracellular matrix around and among fibroblasts was alcian-blue-positive and not fibrous. In the epicardium, neutrophils were infiltrated densely and mixed with fibrin.

By 6–10 days vascular granulation tissue appeared in the peripheral zone of necrosis, and was extending into the residual necrosis progressively (Fig. 4a). The granulation tissue consisted of young fibroblasts with little intercellular fibrous tissue, proliferations of capillaries and infiltration of inflammatory cells. Many macrophages in aggregates surrounded necrosis, and fibroblasts were seen in the outer zone. The stroma of the granulation tissue adjacent to necrosis was stained deep-

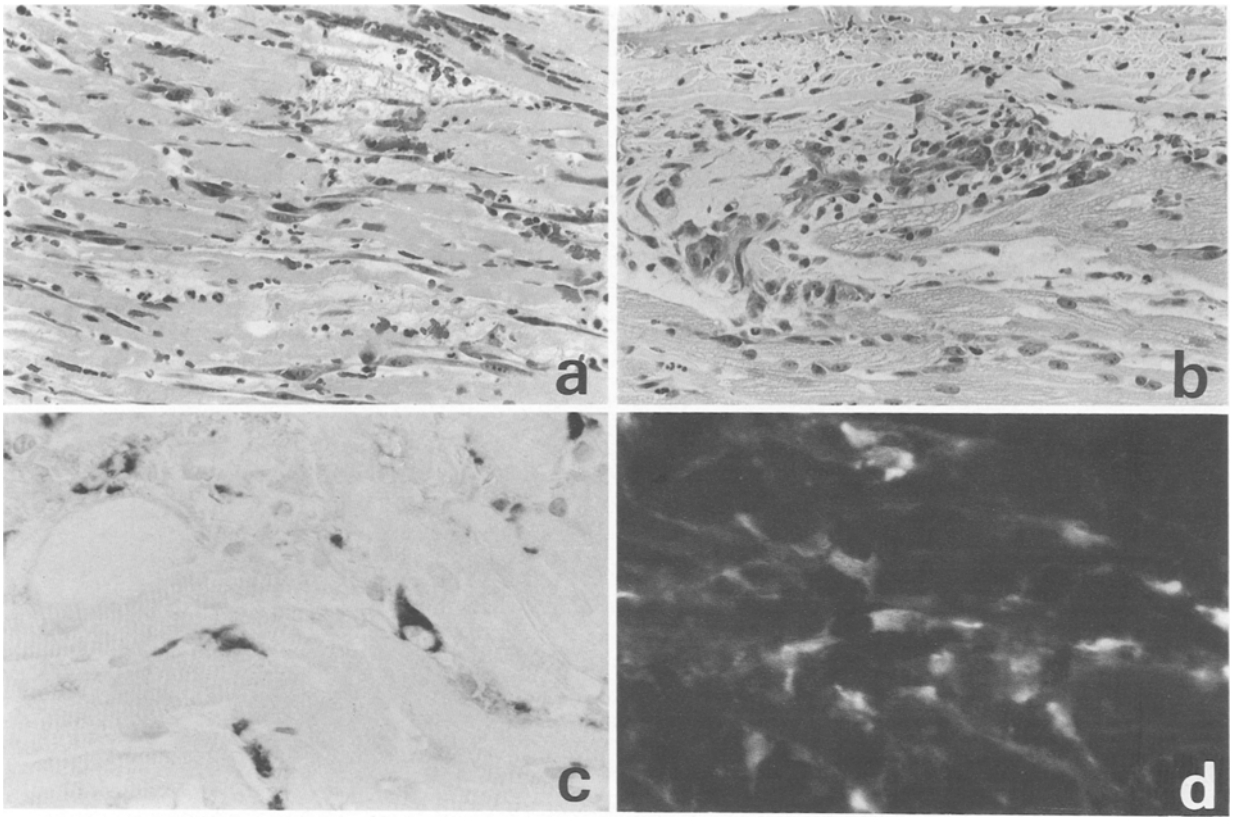

Fig. 3. A wound 3 days after operation. Fibroblasts with prominent nucleoli and plump cytoplasm appear among necrotic myocytes (**a**), and in the endocardium (**b**). Anti-collagen type I (**c**) and type V (**d**) antibodies are reacted with these fibroblasts. **a, b** H & E, ×230; **c** immunoperoxidase, ×470; **d** immunofluorescence, ×300

ly by alcian blue, but collagenous fibres identified by azan stain and silver impregnation were densely packed in the granulation tissue adjacent to the uninjured myocardium. The thrombus adherent to the endocardium and fibrin exudate on the epicardium began to organize, with ingrowth of fibroblasts from the endocardium and epicardium with neovascularization.

A fibrosing granulation tissue phase occurred between 20 and 30 days. The necrotic area of the myocardium was almost replaced by granulation tissue (Fig. 5a). Occasionally small foci of necrosis with immature granulation tissue were still observed. The granulation tissue in this phase was rich in collagenous fibres arranged in parallel, and intervening slender fibroblasts (Fig. 5b). A few lymphocytes, plasma cells and macrophages were scattered around. Organization of the thrombus was almost completed resulting in fibrous thickening of the endocardium. Although the boundary between the endocardium and organized thrombus was blurred on an H & E section, elastica van Gieson stain disclosed the pre-existing elastic lamina of the endocardium which helped to distinguish the fibrous union of the wound from the mural thrombus. Furthermore, it was noted that a small focus of adipose tissue appeared in the fibrosing granulation tissue in proximity to the viable myocardium (Fig. 6). Lipoblasts were seen around the capillaries. In the organized thrombus, there was an area of chondroid tissue which was composed of chondrocytes (Fig. 5f); this area and the chondroid matrix was reactive with anti-collagen type II (Fig. 5g), I and III antibodies.

Adipose tissue was seen at 45 and 90 days when no necrotic foci remained. The subendocardium at the wound was replaced by dense fibrous tissue with a scanty cellular component, and both the myocardium and epicardium were extensively occupied by an adipose tissue instead of a granulation tissue (Fig. 7a). In the organized mural thrombus, there was no adipose tissue. In the entire course of wound healing, the regenerative capacity of the myocytes was apparently poor. Thus, the present ventriculotomy was finally repaired by a scar mixed with an adipose tissue and a dense fibrous tissue.

Immunohistochemically collagen type I was diffusely present in the loose fibrous tissue or perivascular fibrous tissue of the injured endocardium, myocardium and epicardium. Fine reticular fibres among the myocytes were stained faintly. At 3 days after operation, collagen type I appeared in the cytoplasm of proliferating fibroblasts in the wound (Fig. 3c) as well as in the endocardium and epicardium in the boundary zone. Fibroblasts in the granulation tissue growing into the necrotic area were also intensely positive (Fig. 4b, c). The staining intensity gradually decreased with increase of extracellular collagen fibres. The positivity for type I collagen in fibroblastic cells in the endocardium and epicardium was intense up to 10 days. At 30 days, the fibrosing

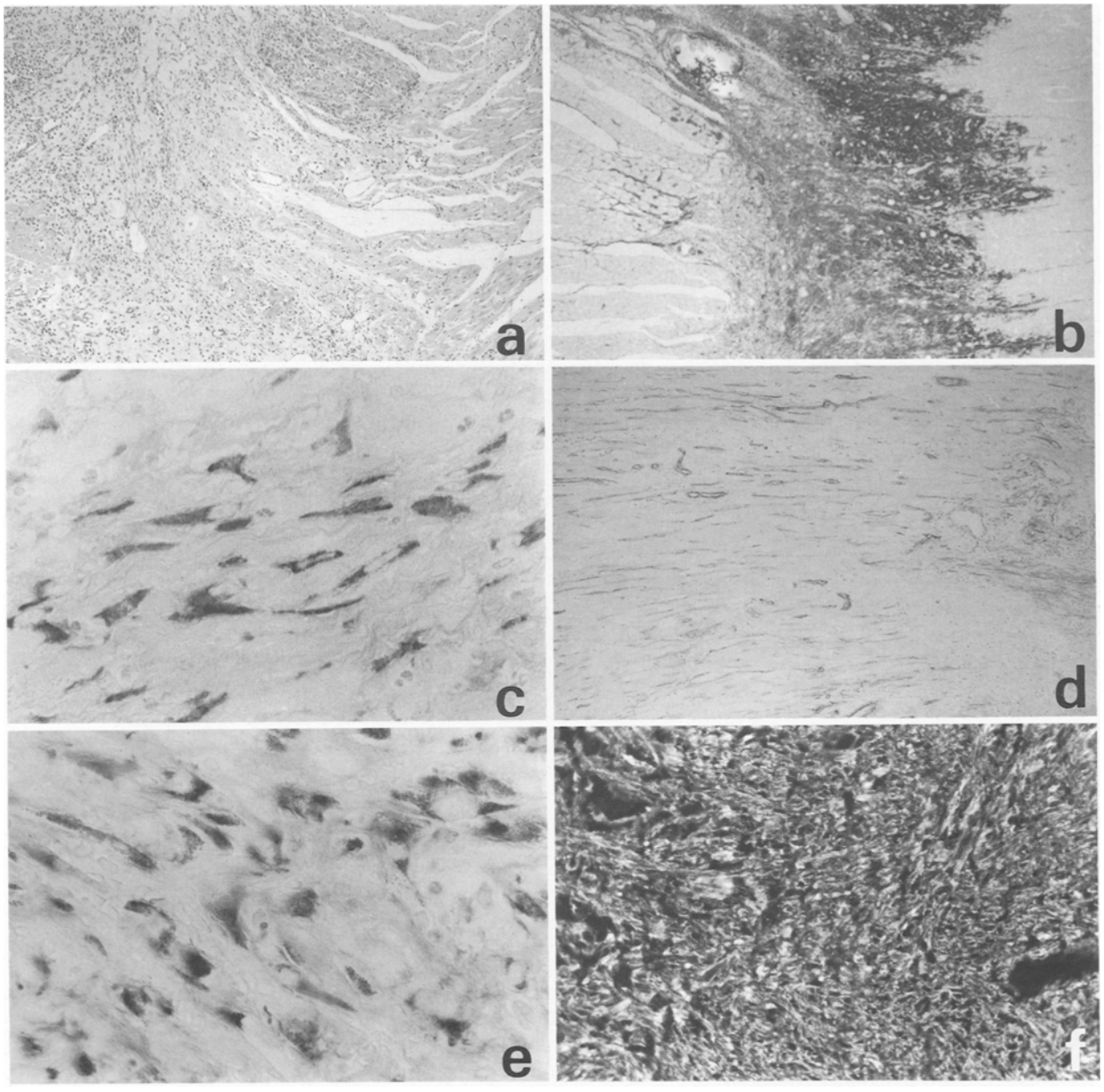

Fig. 4a–f. A wound 10 days after operation. Granulation tissue, composed of loose matrix, fibroblasts and proliferating capillaries, grows into the border of the wound and between areas of necrotic and viable muscle cells (**a**). The loose connective tissue matrix of the wound is positive for anticollagen type I antibodies; newly formed collagen fibres tend to be stained more intensely than the more dense fibrous matrix (centre) (**b**). Cytoplasms of fibroblasts are also positive for anti-collagen type I (**c**) and type V (**e**) antibodies. Basement membrane of myocytes delineated by anti-type IV collagen antibody was invisible at the wound border (**d**). Fibronectin deposited diffusely in the granulation tissue (**f**). **a** H & E, ×60; **b** immunoperoxidase, ×60; **c** immunoperoxidase, ×460; **d** immunoperoxidase, ×60; **e** immunoperoxidase, ×460; **f** immunofluorescence, ×60

granulation tissue and fibrous scar tissue were positively stained for type I collagen (Fig. 5c). At 90 days in the dense fibrous tissue intermixed with adipocytes, collagenous fibres were also positive (Fig. 7b).

During the entire course of wound healing, there was no immunoreactivity to anti-type II collagen antibodies except in the chondroid tissue arising in the organized thrombus 30 days after operation.

Localization of collagen type III was similar to that of collagen type I. Slight differences were that the immunoreactivity to collagen type III in the fibroblasts disappeared slightly earlier than that of collagen type I, and that the staining intensity of collagen type III on newly formed collagen fibres was fainter than that of collagen type I.

In the uninjured portion, mesothelial cells of the epicardium, vascular endothelium, vascular smooth muscle cells and myocytes showed linear and pericellular reactivity for anti-collagen type IV antibodies. The basal lamina scaffold of the myocardium was still preserved in the necrotic area at the 1st post-operative day. At 3 days after operation, the scaffold disappeared with leucocytic infiltration, except around the necrobiotic myocytes in a peripheral zone of the wound. With devel-

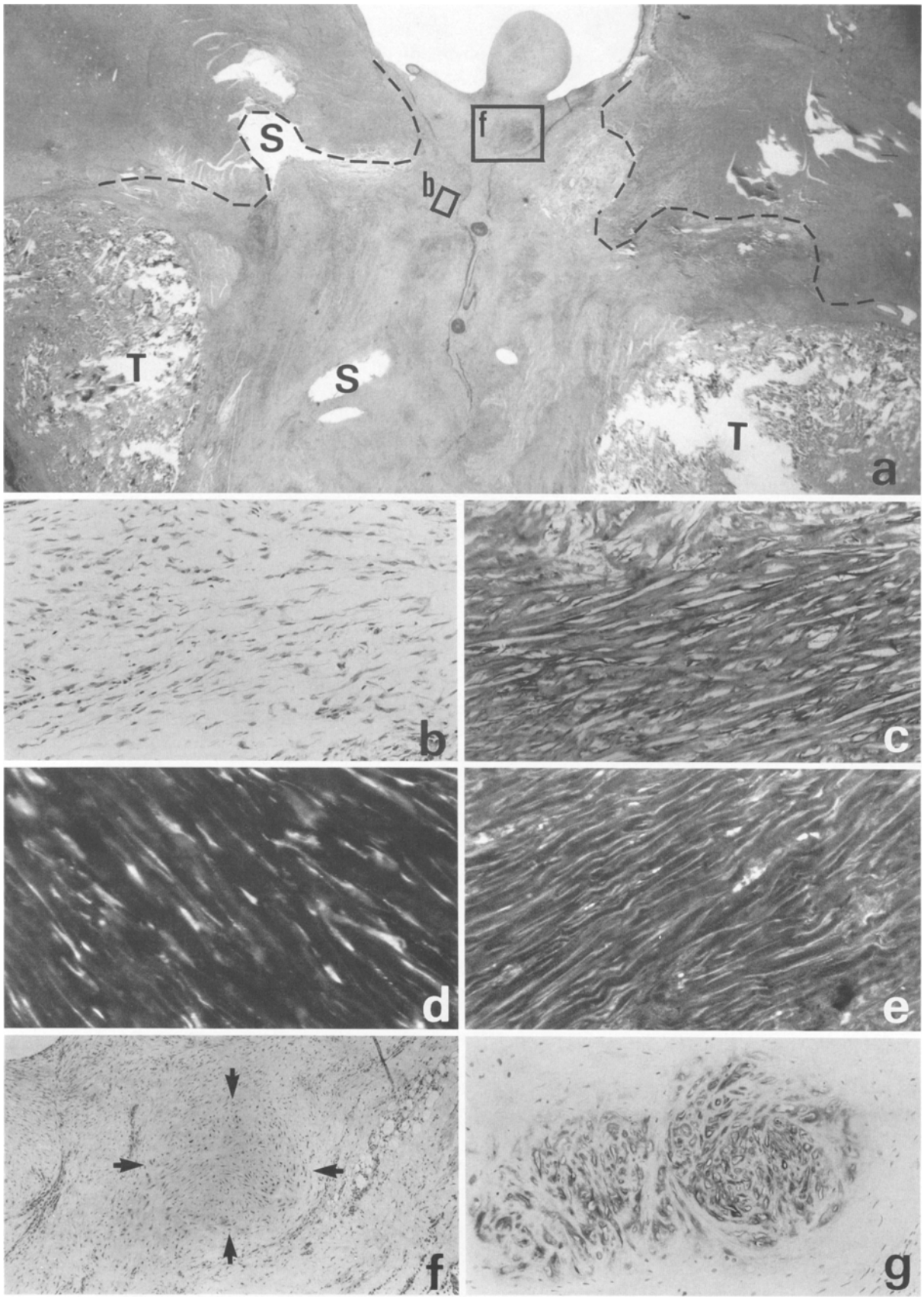

Fig. 5a–g. A wound 30 days after operation. Necrotic myocardium in the wound is replaced by fibrosing granulation tissue (**a**). Squares *b* and *f* are shown close-up in **f**, respectively. *Dotted lines* indicate the boundaries of the intact myocardium. *T* shows areas of Teflon felts and *S* suture sites. The fibrosing granulation tissue is composed of broad bundles of dense collagenous fibres with intervening elongated fibroblasts (**b**), almost all of which are diffusely positive for anti-collagen type I antibodies (**c**). Type V collagen is positive exclusively in the elongated cytoplasmic extension of fibroblasts (**d**). Type VI collagen is positive in a linear pattern among broad fibre bundles (**e**). Chondroid tissue (*arrows*) which develops in the fibrous tissue extending out from pre-existing elastic laminae of the endocardium are indicated by a small square in **a** (**f**) gf is positive for anti-type II collagen antibodies (**g**). **a** H & E, ×4.7; **b** H & E, ×120; **c** immunoperoxidase, ×120; **d** immunofluorescence, ×120; **e** immunofluorescence, ×120; **f** H & E, ×60; **g** immunoperoxidase, ×120

Fig. 6. A small cluster of adipocytes in the marginal zone of wound 30 days after operation. There was no continuity with pre-existing adipose tissue. Lipoblasts in the cluster are recognised around capillaries. H & E, ×60

opment of the granulation tissue, collagen type IV of myocyte origin completely disappeared in the wound (Fig. 4d). Newly formed capillaries in the granulation tissue were delineated by collagen type IV. It was also positive around adipocytes at the wound in the terminal stage of healing.

As for type V collagen, there was no immunoreactive product in the heart evidenced by immunofluorescence or immunoperoxidase methods. Type V collagen was detected immunohistochemically at 3 days after operation in the plump cytoplasm of fibroblasts in the boundary zone between necrotic and surviving myocardium (Fig. 3d), and in the endocardium and epicardium (Fig. 4e). Elongated fibroblasts in the later phase and the intercellular collagenous fibres along their cytoplasmic extensions also showed positivity (Fig. 5d). At 90 days, type V collagen-positive fibroblasts disappeared

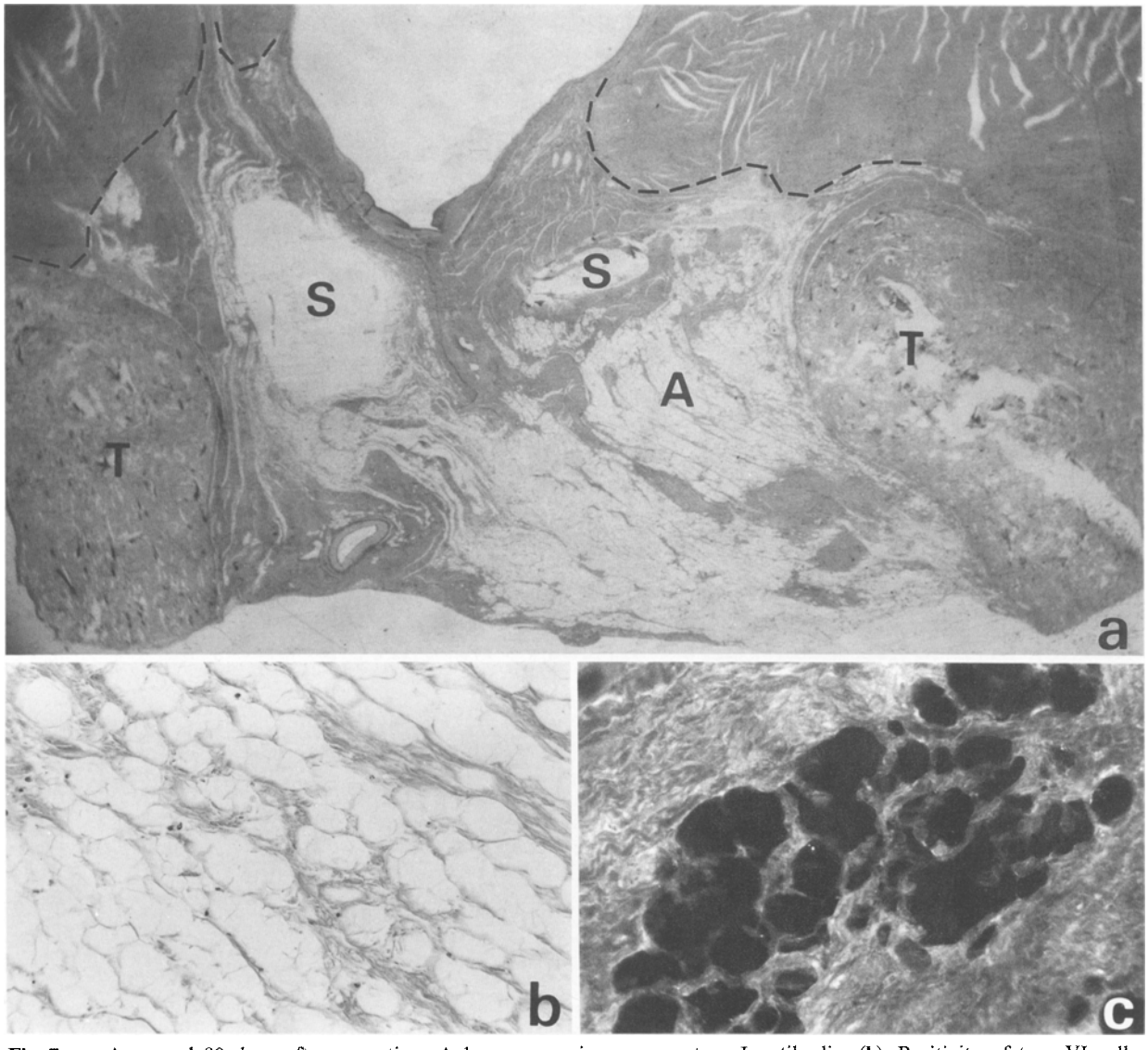

Fig. 7a–c. A wound 90 days after operation. A low-power view of the wound showing almost entire replacement by an adipose tissue (*A*) except for a thin dense fibrous scar tissue at the endocardial-aspect (**a**). Fibro-adipose scar tissue is positive for anti-collagen type I antibodies (**b**). Positivity of type VI collagen is noted in a linear pattern in the dense fibrous scar tissue of subendocardium (**c**). **a** H & E, ×4.7; **b** immunoperoxidase, ×60; **c** immunofluorescence, ×60

Table 1. Extracellular matrices of the heart wound evaluated by immunoperoxidase and immunofluorescence

Days after wounding	1–3	6–10	20–30	45–90
Collagen, types I and III	−(+)	+(++)	++(+)	++
Collagen, type V	−(+)	−(++)	+(++)	−
Collagen, type VI	−	−	+	+
Fibronectin	++	++	+	+

The immunopositive areas are graded on a scale of −, + and ++. Parentheses show the cytoplasmic positivity of the fibroblasts

from the dense fibrous tissue of the subendocardium and in the adipose tissue replacing the myocardium at ventriculotomy.

Anti-collagen type VI antibodies showed a linear staining pattern in the fibrous tissue of the endocardium and epicardium at ventriculotomy. Weak linear positivity was also observed in the interstitium of myocardial fibres and blood vessels in the fibrosing granulation tissue phase (Fig. 5e). Such a linear positivity persisted up to 90 days in the fibrous scar tissue and in the adipose tissue (Fig. 7c). No intracytoplasmic positivity was found during the entire course.

Fibronectin was usually localized in the perivascular and inter-myocardial spaces and also in the endocardial and epicardial loose fibrous tissue. At ventriculotomy, a large mass of exudate in the epicardium and the thrombus showed intense immunofluorescence. Fibronectin appeared diffusely in the vascular granulation tissue (Fig. 4f), and in the fibrosing granulation tissue and fibrous tissue weak linear positivity remained between collagen fibre bundles. There was neither intracytpolasmic positivity of fibronectin nor immunoreactivity in the adipose tissue during the experimental course.

A summary of the immunohistochemical findings is shown in Table 1.

Discussion

It is interesting that the present ventriculotomy finally healed as a fibrous scar admixed with extensive adipose tissue. Warren et al. (1957) described similar fatty infiltration in the healing of large right ventriculotomy. Such fatty infiltration in the heart has been known to occur in human cases of myocarditis, dysplasia of right ventricle (Iwa et al. 1988) and Uhl's anomaly (Uhl 1972). These are all in the right ventricle. Several authors (Carter and MacMillian 1950; Dillon and Postlethwait 1961; Thomas et al. 1952; Frankel et al. 1961) have reported that longitudinal incision in the left ventricle of the dog has resulted in an ordinary fibrous scar, unlike the present results. Examination of these texts, however, shows that adipose tissue was demonstrated in the fibrous scar.

It is well-known that tissue remodelling is an important phenomenon in the reparative process of specialized organs. During the repair of bone fractures, provisional callus or primary callus is absorbed and remodelled, and in carrageenin granuloma becomes fibrotic and is subsequently almost replaced by mature adipose tissue (Fisher and Paar 1960). In skeletal muscle injury, loss of muscle is replaced by other connective tissues, initially consisting of fibroblasts, gradually followed by adipocytes probably associated with contraction of the striated muscle itself (Carpenter and Karpati 1984). It is thus assumed that pressure on the cardiac muscle and wounds of long duration stimulate the proliferation of adipocytes after fibrous union is formed.

The tensile strength of fibrous scars in the heart is reportedly greater than that of viable myocardium (Frankel et al. 1961). In the present study, the fibrous scar that was formed in subendocardium is presumably tough enough to resist the cardiac pressure load. The rest of the granulation tissue at the wound may be remodelled and replaced by adipose tissue. Increase in tensile strength in wound healing is usually related to the covalent cross-linking of collagen molecules, probably of type I and III (Forrest and Jackson 1971; Bailey et al. 1975). In the present experiment, it was noted that type VI collagen was recognized increasingly in the thick collagen fibre bundles which were intensively immunoreactive with anti-type I and type III collagen antibodies. Collagen type VI is quite different from the other interstitial collagens in its high content (70%) of non-collagenous protein (Rauterberg 1986) and in its aggregates which form microfilaments less than 10 nm in diameter (von der Mark et al. 1984; Gibson and Clear 1983). Collagen type VI is distributed widely in the body, particularly in dense fibrous connective tissues such as a tendon, dermis and ligamentum nuchae usually appearing in a thin linear pattern among thick collagen fibre bundles. Mayne et al. (1986) claimed that type VI collagen fibres preserve the structure of dense fibrous tissue. It presumably plays an important role in structural organization of wounds and fibrous unions by inter-connecting thick fibre bundles.

In the early phase of the present experiment fibroblasts were reactive with anti-collagen types I, III and V antibodies. Intracellular positivity suggests active production of collagen types I, III and V. Cytoplasmic staining intensity of type I and III becomes fainter as interstitial collagen deposition is evident. These results agree with a culture study showing that collagen production of cultured fibroblasts gradually decreased from a maximum after 2 or 3 days of culture (Rennard et al. 1980). A biochemical quantitative analysis reveals that the III/I ratio increases in the early phase of wound healing in comparison with normal tissue, and decreases gradually with maturation of the wound (Shuttleworth 1975). The present immunohistochemical study clearly showed co-distribution of types I and III, although the quantitative differences were not examined.

Collagen type V appeared in fibroblasts in the entire period of wound healing, together with types I and III. The co-production of different collagen types has been shown biochemically by Hering et al. (1983). In the scar phase of the present experiment, unexpectedly, collagen type V was not demonstrated in fibrous tissue. In gener-

al, collagen type V increases relatively in chronic fibrosing states such as arteriosclerosis (Ooshima 1981), hypertrophic scars (Narayanan and Page 1985) and lung fibrosis (Madri and Furthmayr 1980). Thus, there seems to be a discrepancy between production and deposition. Collagen type V molecules generate a hybrid fibril with type I collagen molecules in vitro (Adachi and Hayashi 1986). Recently, these two molecules were shown to be in a single collagen fibril in vivo (Birk et al. 1988). Thus, negative staining of type V collagen in thick fibre bundles themselves may be due to few antigenic domains being exposed in tissue sections. Collagen type V may be reacted with its antibodies after collagen fibres are swollen with acetic acid (Linsenmayer et al. 1983).

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