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Angiogenesis and expression of tenascin after transmural laser revascularization

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Summary. Transmyocardial revascularization (TMR) with CO_2-laser equipment is an alternative approach in the treatment of patients with severe ischemic cardiac disease. Several studies concerning morphological features after TMR document a strong transmyocardial injury, but little is known about wound healing in laser-induced alterations of the cardiac skeleton and their putative role for angiogenesis and endothelialization. The present study was conducted to establish a useful immunohistochemical marker for detection of these laser-induced injuries and to analyze starting points of angiogenesis in human myocardium after TMR. Our data show that tenascin labeling is a useful immunohistochemical approach to detect laser-alterated segments of the cardiac skeleton as well as laser-induced fibrosis. Starting points of the angiogenetic process are seen throughout the margins of laser-induced lesions, where myocardial capillaries are found. Disrupted vessels located within laser-alterated connective tissue septa are not major starting points for endothelialization of laser-induced lesions and for capillary sprouts. In comparison to laser-induced fibrosis, induction and promotion of angiogenesis by laser radiation is weak.

Key words: Angiogenesis, Cardiovascular disease, Laser, Revascularization

Introduction

The use of transmyocardial laser revascularization (TMR) in order to revascularize ischemic myocardium by means of directing oxygenated left ventricular blood into myocardial tissue is a new approach in treatment of patients with severe ischemic cardiac disease (Frazier et al., 1995; Horvath et al., 1997). The creation of transmyocardial channels from the epicard to the left ventricular cavity with high-energy CO_2-laser radiation was first reported by Mirhoseini and coworkers (Mirhoseini and Cayton, 1981; Mirhoseini et al., 1982).

Findings from extensive animal trials support the hypothesis that these endothelialized channels are linked to a newly formed capillary network and that a systolic blood flow from the left ventricular cavity into the ischemic myocardium could be established (Mirhoseini and Cayton, 1981; Whittaker et al., 1996). It is assumed that the angiogenetic process starts after TMR beginning in laser-disrupted vessels within septa of the cardiac skeleton (Cooley et al., 1994; Krabatsch et al., 1997; Schweitzer et al., 1997).

In humans, the morphological basis for TMR-induced effects, as well as reasons for response or non-response from TMR treatment, are not well understood. Several human studies concerning morphological features after TMR have documented strong zonal alterations of the myocardium adjacent to laser-induced lesions (Cooley et al., 1994; Gassler et al., 1997; Moosdorf et al., 1997; Schweitzer et al., 1997), but little is known about disruption of the cardiac skeleton or angiogenesis after transmural laser treatment.

The present study was conducted in order to establish a useful immunohistochemical marker for the detection of laser-injured septa and to analyze the location of starting points of angiogenesis in human myocardium after TMR.

Materials and methods

Patients

Clinical and anamnestic data for the six patients treated with TMR is given in the Table 1. In all patients, grade IV angina pectoris was diagnosed according to CCS (Canadian Cardiovascular Society) grading before and after TMR treatment. Grading is defined as follows (Goldman et al., 1981): grade I: ordinary physical activity does not cause angina; grade II: slight limitation of ordinary physical activity; grade III: marked limitation of ordinary physical activity; grade IV: inability to carry on any physical activity without discomfort; anginal syndrome may be present at rest. In all patients, the Heart Laser System (PLC Medical

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Table 1. Clinical and anamnestic data for the patients treated with TMR.

PATIENT	SEX	AGE (years)	No. OF LASER APPLICATIONS	TOTAL ENERGY OF OF LASER PULSES (J)	DEATH AFTER TMR (days)	CAUSE OF DEATH
1	F	67	35	1225	3	Relapsing myocardial infarct
2	M	55	38	1520	3	Left ventricular failure
3	M	60	31	1240	7	-*
4	F	63	35	1400	16	Relapsing myocardial infarct
5	M	52	30	1200	90	-*
6	F	48	31	1240	150	Left ventricular failure (sudden death)

TMR indicates transmyocardial laser revascularization. *: heart transplantation was performed after TMR treatment and the explanted heart was investigated.

Systems, Inc.) was used with 1000W/mm^2 CO_2-laser equipment. A protocol for application of 1300J energy per heart was performed. Thus, about 33 transmural laser pulses were distributed over the whole free left ventricular wall using the surgical technique, as was previously described (Smith et al., 1995).

Morphological studies and immunohistochemistry

Investigations of hearts were performed as previously recorded (Gassler et al., 1997). Briefly, after fixation of the whole organ in 10% paraformaldehyde (PFA) for 48h, transmural blocks that included the laser-induced lesions were excised, postfixed overnight at 4 °C in 4% PFA, embedded in paraffin and cut crosswise or lengthwise. For light microscopic evaluation serial sections were prepared (each section about 5 μm). Serial sections for histomorphological investigations were stained with trichrome (Masson-Goldner). Representative sections were selected and used for immunohistochemistry. All sections were observed in a Polyvar 2 (Reichert-Jung, Vienna, Austria).

Antibodies

Anti-human collagen types I (1:100) and III (1:100) polyclonal antibodies elicited in rabbit (Quartett), monoclonal antibodies from mouse against human collagen type IV (1:400), type VI (1:100), desmin (1:200), laminin (1:1000) and tenascin (1:400), respectively (all from Dako), anti-vimentin monoclonal antibody elicited in mouse against swine vimentin (1:50; Dako), anti-human fibronectin polyclonal antibody raised in rabbit (1:1000; Quartett), monoclonal CD31 (undiluted antibody; Biogenex), the bridging antibodies from Dianova as well as Dako and the alkaline phosphatase-antialkaline phosphatase (APAAP) complex (Progen) were used.

Immunohistochemistry

For immunohistochemical procedures, representative sections were deparaffinized and sequentially treated as follows. For collagen types I, III, IV, VI, CD31, desmin, fibronectin and tenascin staining, sections were pretreated by 0.05% protease type XXIV (Sigma) for 15min at 37 °C, for laminin staining by both 0.2% hyaluronidase (Sigma) for 30 min at 37 °C, followed by 0.05% trypsin (Sigma) for 3 min at 37 °C. Enzymatic pretreated sections and sections for vimentin staining were incubated with primary antibodies for 1h at 37 °C; and sections for laminin staining overnight at 4 °C. All sections were immunostained by the APAAP method (Cordell et al., 1984) and counterstained with hemalum. For all studies, negative controls were performed with substitution of the primary antibody with PBS or blocking solution. Because of the nonquantitative nature of immunohistochemistry, the relative staining intensity of the studied antigens was scored twice by two investigators and designated with a profile: -, no staining; +, minimal; ++, mild; +++, moderate; ++++, marked.

Results

The cardiac skeleton at days three and seven after TMR

In laser-induced lesions a fibrinous network, erythrocytes, granulocytes and thrombocytes as well as cellular debris were seen. Residual collagenous septa never crossed these transmural defects, indicating that the cardiac skeleton was fully disrupted there by laser radiation. Blunt ends of injured collagenous septa were found to be located at the edges of laser-created wounds. In these septual structures the collagenous fibers were only loosely attached to each other and infiltrated by erythrocytes and other blood-derived cells. Clots and cellular detritus were formed (Fig. 1). Strong extracellular anti-tenascin staining - that was neither seen in normally structured septa nor in cardiac fibrosis - was regularly found in injured segments of cardiac septa (Fig. 2). Serial sections revealed that small vessels, i.e. arterioles and venules, within these septa were injured (Fig. 3). Only endothelial cells of these vessels were positively stained with the anti-CD31 antibody, but neither evidence for a migration of endothelial cells nor capillary sprouts were found. The margins of laser-induced lesions were negative for CD31, including the funnel-shaped and sealed epicardial and endocardial defects. In comparison to laser-untreated myocardium, differences in the expression of collagen types I, III, IV

and VI as well as desmin, fibronectin, laminin or vimentin were not given.

The cardiac skeleton at day 16 after TMR

In laser-created lesions a heterogenous matrix was established containing a fibrinous network, erythrocytes, thrombocytes, macrophages and few inflammatory cells. Compared to the findings at days three and seven, the infiltration of injured septa with blood-derived cells was reduced. Immunohistochemically, the whole cardiac skeleton was strongly stained for collagens type I and III, but a positive signal for tenascin was only found in the injured septual segments adjacent to laser-induced

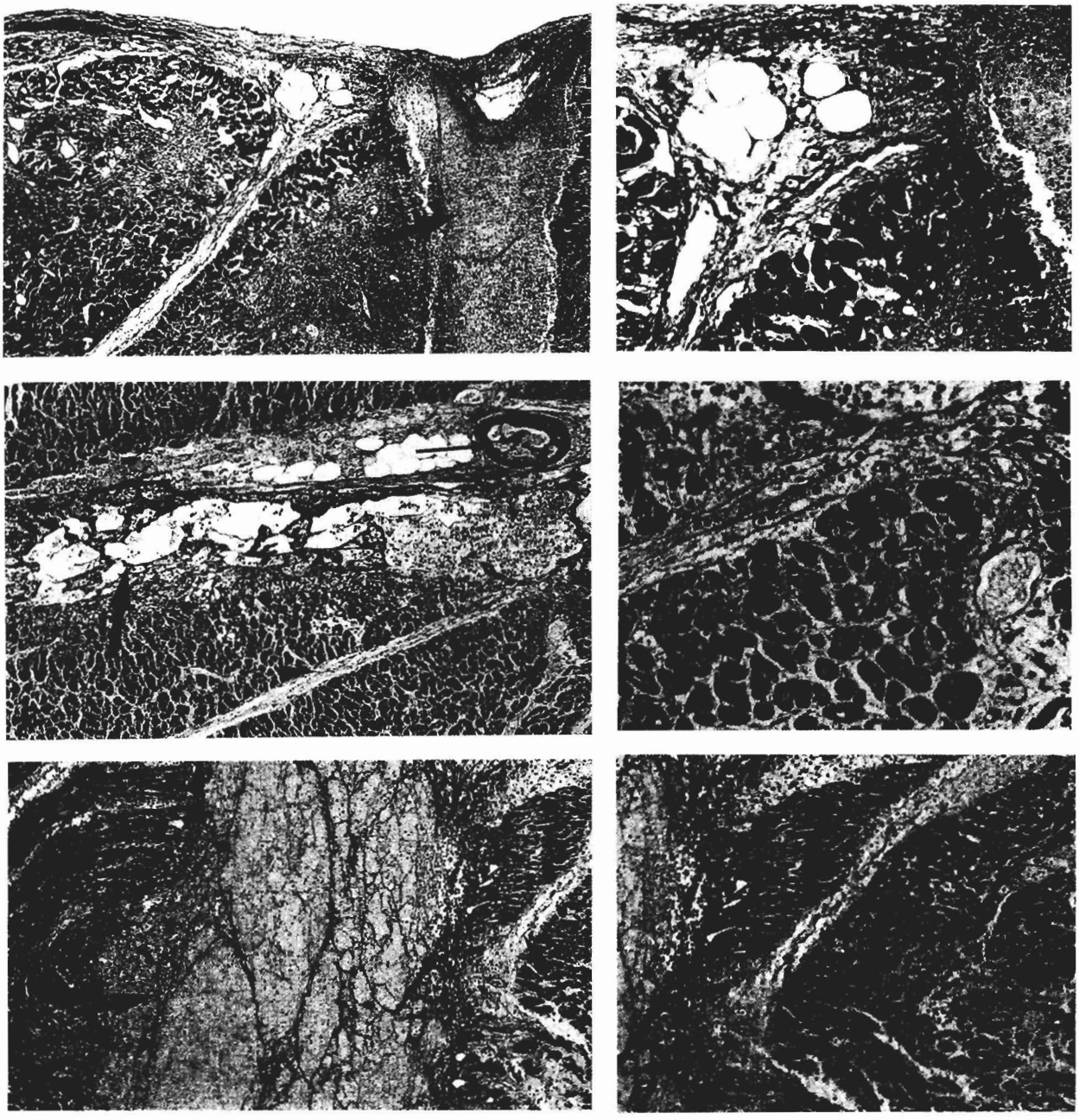

Fig. 1. Histological features of laser-induced injury in human myocardium three days after TMR. **A.** Endocardial aspect of disrupted connective tissue septa (arrowhead) and laser-created lesion (arrow). Trichrome Masson-Goldner. x 40. **C and E.** Injured connective tissue septa (arrowheads) are located adjacent to transmural laser-induced lesions (big arrow). A clotted intramyocardial arteriole is shown in panel C (small arrow). Trichrome. x 60. **B, D and F.** Laser-induced damage of the cardiac skeleton at higher magnification. Trichrome. B, D, x 200; F, x 80

transmural lesions. In the interface of both structures capillary sprouts were not seen. The expression of CD31 within connective tissue septa was found to be restricted to the endothelium of these vessels. At the margins of laser-induced lesions, endothelium-like cells - positively stained for anti-CD31 - were occasionally observed.

The cardiac skeleton at days 90 and 150 after TMR

In serial sections, all laser-induced lesions were found to be scarred without the development of patent and endothelialized channels, but small vessels and capillaries were embedded in the collagenous matrix. These endothelialized structures - positively stained for CD31 - were directly linked to small patent intramural vessels. Thus, a capillary network consisting of these small vessels and endothelialized structures in formerly laser-induced lesions was established (Fig. 4). The capillaries were stained for collagen type VI, the endothelial cells for CD31. Immunohistochemically, a strong string-like expression of tenascin was regularly seen in the interface of original cardiac tissues and scarred transmural laser-created lesions, forming a characteristic double line pattern (Fig. 2). In these fibrosed structures, a fibrillar expression of collagen types I and III as well as a granular distribution of fibronectin and vimentin were found. The expression of collagen type IV and laminin were restricted to basement membranes, and the expression of desmin was exclusively seen in the cytoplasm of cardiocytes. Adjacent to

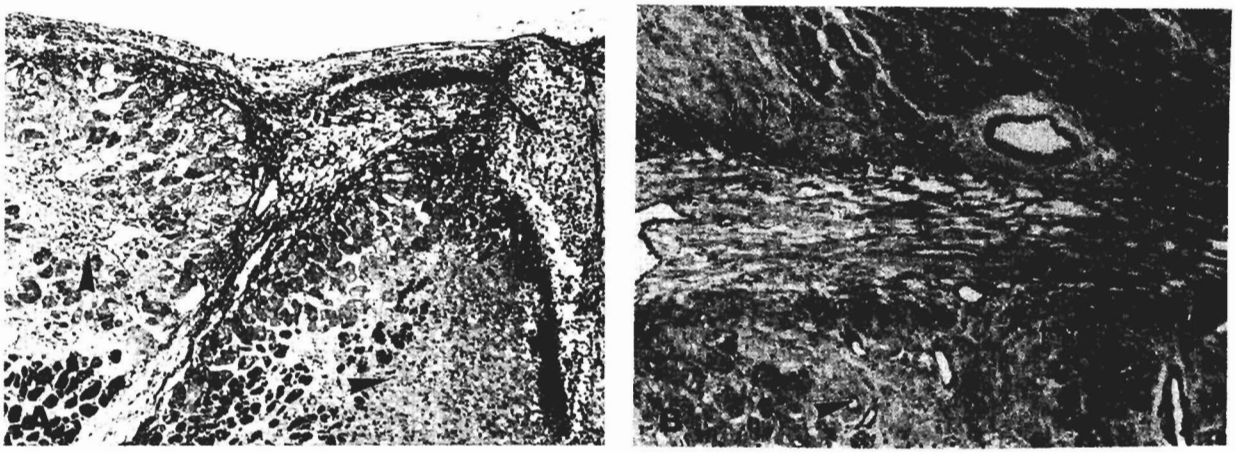

Fig. 2. Immunohistochemical analysis of tenascin expression in laser-created lesions at day three (A) and day 90 (B) after TMR. Anti-tenascin staining with hemalum counterstain. **A.** Expression of tenascin is exclusively found in laser-injured septa (short arrows), but not in cardiac fibrosis (arrowheads). The transmural laser-induced lesion is marked by a long arrow. x 60. **B.** In laser-induced fibrosis, expression of tenascin is found in a characteristic double line pattern (short arrows). Cardiac fibrosis (arrowhead) as well as connective tissue septa (arrow) are not stained. x 80

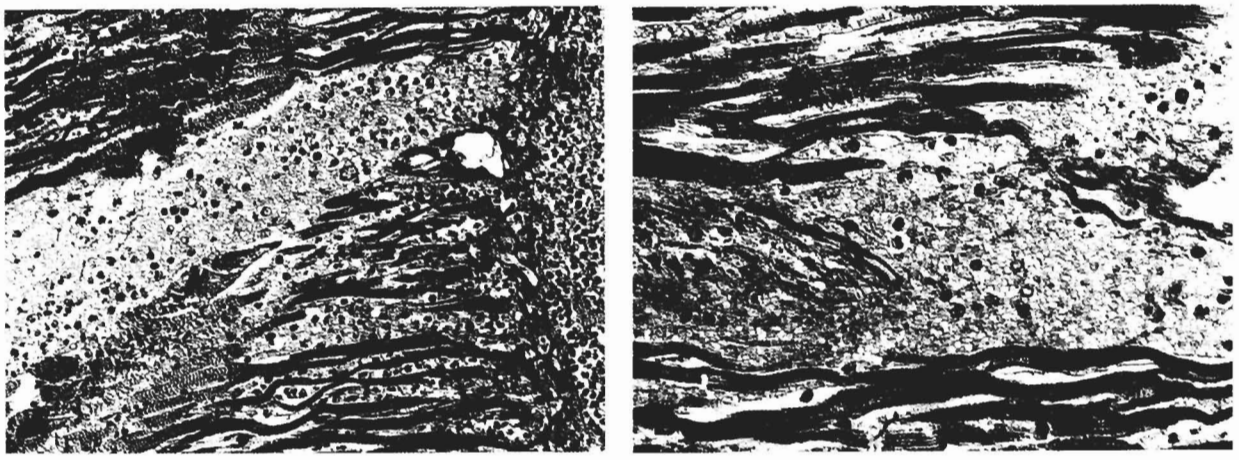

Fig. 3. Histological features of laser-damaged septa at day 3 after TMR. **A.** The link between a transmural laser-created lesion (right) and a laser-damaged septum is shown. Inflammatory cells and erythrocytes migrate into the injured tissue. Note cardiocytes with distinct contraction bands. Trichrome Masson-Goldner. x 160. **B.** Laser-injured septum in higher magnification. Remnants of the collagenous matrix are infiltrated by erythrocytes and blood-derived cells. Trichrome Masson-Goldner. x 240

laser-induced lesions, cardiocytes were found to be separated from each other by scarred tissue (Fig. 4).

Discussion

TMR is a new therapeutical approach in patients with severe ischemic cardiac disease in addition to medical and surgical treatment (Frazier et al., 1995; Horvath et al., 1997). Clinical results suggest that TMR may improve anginal status, relative endocardial perfusion and cardiac function in patients who do not suffer from preoperative congestive heart failure (Frazier

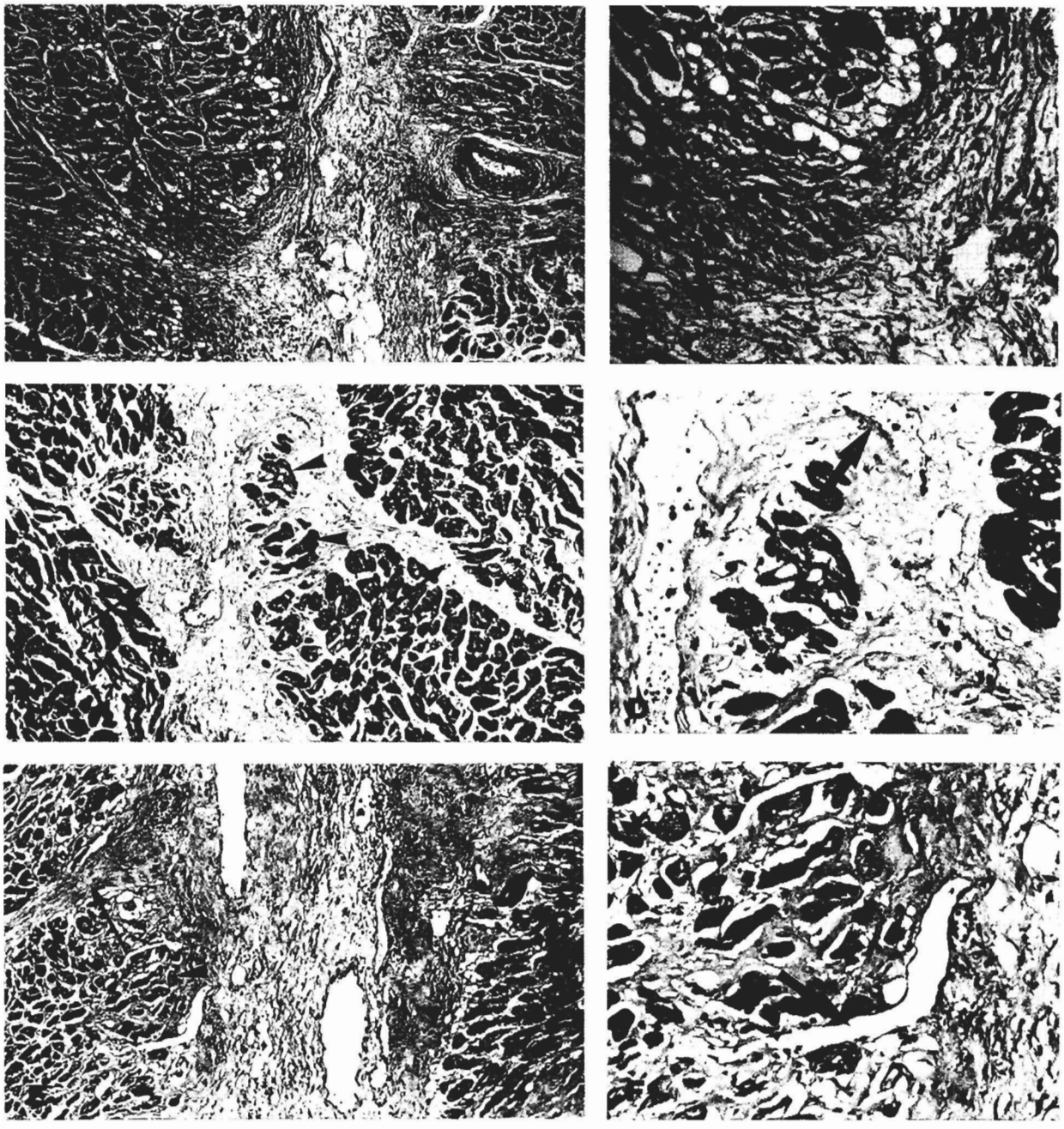

Fig. 4. Laser-induced fibrosis at day 150 after TMR. **A, C and E.** Intramyocardial fibrosis consisting of scarred original laser-induced lesions and fibrosed septa (arrow) of the cardiac skeleton. C. Cardiocytes surrounded by the extended laser-induced fibrosis are separated from each other (arrowheads). Trichrome Masson-Goldner. x 80. **B, D andF.** Laser-induced fibrosis of the cardiac skeleton at higher magnification. Septual vessels (B,D; arrow) as well as intramyocardial vessels (F; arrow) are linked to the capillary network in scarred laser-created lesions, endothelial cells are clearly distinguished. Trichrome Masson-Goldner. x 200

et al., 1995; Donovan et al., 1997). The improvement of perfusion by transmural lasering is probably based on a newly formed intramural capillary network (Fisher et al., 1997; Krabatsch et al., 1997) and/ or a systolic blood flow from the left ventricular cavity into ischemic myocardium (Mirhoseini and Cayton, 1981; Hardy et al., 1990; Whittaker et al., 1996). It is assumed that angiogenesis - creating the capillary network - starts after TMR beginning in laser-disrupted septa. Putative mechanisms for laser-induced injury of the cardiac skeleton include: (i) the absorption of laser-derived energy; and (ii) shearing stress from cardiac work probably intensified the initial septual defect resulting in a zipper-like septual disconnection and laceration. Laser-induced injury of the cardiac skeleton and loss of cardiocytes are suggested to influence the outcome after TMR (Schweitzer et al., 1997). The present study was conducted to establish an immunohistochemical marker for the detection of laser-disrupted septa in humans and to evaluate laser-induced angiogenesis. Therefore, TMR-treated human cardiac tissues were investigated using a morphological and immunohistochemical approach.

Tenascin is known as a marker of damaged tissues because of its importance for epithelial-mesenchymal interactions, rapid cell movement and adhesion-modulating activities (Chiquet-Ehrismann et al., 1991; Whitby et al., 1991). Strong expression of tenascin, but not of vimentin, was seen in laser-damaged connective tissue septa, early after TMR. In laser-induced fibrosis, expression of tenascin was detected in a characteristic double line pattern, probably displaying formerly margins of laser-induced lesions. Our data show that tenascin is exclusively found in laser-created injuries of the cardiac skeleton, but not in ischemia-induced tissue alterations. Reasons for the expression of tenascin after TMR have not been investigated by the technique used here and are topics worthy of future consideration. A thermally and/or pressure-mediated effect on fibroblasts to express tenascin can only be hypothesized. Data about expression of tenascin during wound healing after conventional cardiothoracic surgery, i.e. ventriculotomy, have not been published, yet. In laser-induced fibrosis, expression of collagen types I, III, IV, VI as well as fibronectin, laminin and vimentin was not different to the findings in cardiac scars of other origin (Medugorac, 1982; Kawahara et al., 1990). It is supposed that the quantitative relation between collagen types I and III is important for optimal dynamics in the cardiac cycle and the tensile strength of fibrous scars. Regularly, cardiocytes were found to be separated by laser-induced fibrosis, which indicates conductivity disorders and regional dysfunction (Marino and Zardini, 1997). In conclusion, one can say that tenascin is exclusively and highly expressed in laser-injured connective tissue septa. Immunohistochemically, the pattern of expression apparently depends on the progress in wound healing after TMR.

Laser-induced injury of the cardiac skeleton was established by morphological features and the characteristic anti-tenascin staining (see above). Our morphological and immunohistochemical data show that disrupted vessels of laser-alterated tissue septa are not major starting points of angiogenesis. Angiogenesis and endothelialization apparently start from myocardial capillaries located adjacent to laser-created lesions. In comparison to laser-induced fibrosis, induction and promotion of angiogenesis by laser radiation is weak. As shown by Moosdorf and coworkers, angiogenetic factors are able to stimulate the angiogenetic process after TMR, but the clinical relevance is in discussion (Moosdorf et al., 1997). To summarize our data concerning angiogenesis after TMR, laser-disrupted vessels within connective tissue septa are apparently of low importance for the creation of the capillary network that is usually found after transmural lasering.

In summary, we have shown that anti-tenascin labeling is a useful immunohistochemical approach to detect laser-alterated segments of the cardiac skeleton and laser-induced fibrosis. Induction and promotion of angiogenesis by laser radiation are of low sufficiency. Starting points of the angiogenetic process are apparently located throughout the margins of laser-induced lesions, where myocardial capillaries are found. In laser-alterated connective tissue septa, disrupted vessels are not major starting points for endothelialization of laser-induced lesions.

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