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ORIGINAL ARTICLE

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A comparative study on the immunohistochemical detection of early myocardial damage

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Abstract The study was undertaken to evaluate the kinetics and distribution patterns of several immunohistochemical markers in ischemically and hypoxically damaged myocardium. The myocardium of 8 cases of acute myocardial infarction (AMI), 8 cases of diagnosed acute cardiac death (ACD) and 12 cases of acute exogenic hypoxia (AEH) due to CO poisoning or hanging were analysed for depletion of the cardiac antigens FABP, troponin C and T, desmin and myoglobin, loss of CD59 and deposition of the plasma antigens fibrinogen, fibronectin and the terminal complement complex C5b-9. The visualisation of the terminal complement complex was positive as early as 30 min after onset of symptoms of AMI. Depletion of cellular antigens started earlier than the deposition of plasma antigens. The deposition of fibronectin and fibrinogen began earlier than the detection of C5b-9 but later than the depletion of the cellular antigens. Our findings indicate that for the immunohistochemical detection of very early myocardial damage, the depletion of myoglobin is at least of the same rank or better than depletion of FABP and troponin.

Key words FABP · Troponin · Myoglobin · Fibronectin · Myocardial infarction

Introduction

Using conventional histochemical staining procedures an acute myocardial infarction (AMI) can only be detected 4–6 h after the onset of ischemia (Scotti and Hackel 1985). Myocardial damage can be detected earlier by enzyme histochemistry, especially by the visualisation of dehydrogenases as well as electron microscopy, however many artefacts that simulate vital changes can result from autolysis and the methods are not easily performed on a routine basis (Knight 1973; Leadbetter et al. 1989).

More recently, a series of immunohistochemical markers has been introduced. C5b-9 was judged to be specific for necrosis which allowed detection of single-cell damage and the specificity was not reduced due to putrefaction (Thomsen and Held 1994). This antigen can detect early myocardial injury 40 min after the beginning of the ischemic process (Thomsen and Held 1995). CD59 inhibits the cytolytic activity of complement and thus its loss can be expected before C5b-9 becomes positive (Vakeva et al. 1994). Other markers such as myoglobin, desmin, fibrinogen and fibronectin, have been investigated by various authors (Brinkmann et al. 1993; Amberg 1995). The early postischemic detection of cardiac proteins in serum, especially troponin (Fitzgerald et al. 1996) and the heart-type fatty acid binding protein FABP (Kleine et al. 1992; Glatz et al. 1994) suggests that these proteins are more suitable than heart-type creatine kinase. Troponin is a contractile protein which comprises 5% of muscle proteins and has three subunits which consist of a globular end and a rod. The globule is a complex of the 21-kD troponin I and the 18-kD troponin C, while the rod is composed of the 66-kD protein troponin T (Dhoot et al. 1979). FABP is a 15-kD non-enzymatic protein possibly involved in the intracellular transport of lipophilic compounds and is found in high concentrations in the heart (Borchers et al. 1989). Its early depletion from cardiomyocytes was recently demonstrated (Kleine et al. 1993) by unchanged staining intensities with the H&E and NBT (nitroblue tetrazolium) methods.

In this study, we compared these recently reported markers with established ones to elaborate the efficacy for the detection of early ischemic lesions.

Materials and methods

The study was carried out on paraffin tissue blocks from 28 autopsies performed between 1995 and 1997. The postmortem intervals varied from 8 to 96 h (Table 1) and the tissue samples were fixed

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Table 1 Panel of antibodies investigated

Antibody against	Autoclave pre-treatment	Proteinase K pre-treatment	Incubation time of primary antibody, temperature	Concentration of primary antibody
Fibrinogen (DAKO)	–	–	60 min, 20 °C	1 : 2000
Fibronectin (DAKO)	–	30 min	30 min, 20 °C	1 : 1000
Desmin (Sigma)	+	10 min	60 min, 20 °C	1 : 40
Myoglobin (DAKO)	+	10 min	60 min, 20 °C	1 : 500
C5b-9 (DAKO)	+	10 min	16 h, 4 °C	1 : 25
CD59 (Pharmingen)	+	10 min	60 min, 20 °C	1 : 25
Troponin T human fast muscle (Novo Castra)	+	–	16 h, 4 °C	1 : 10
Troponin C human cardiac (Novo Castra)	+	–	60 min, 20 °C	1 : 100
FABP heart type	+	10 min	60 min, 20 °C	1 : 400

for 1-7 days in buffered formalin. The cases could be divided into three groups (Table 1 a–c) as follows:

Group 1: Eight cases of acute myocardial infarction (AMI) either visible by gross examination (n = 5) or with conventional histology alone (n = 3) using H&E, Luxol fast blue (Arnold et al. 1985) and Lie staining methods (Lie et al. 1971). The latter two staining procedures were used as screening methods only and if positive, a corresponding H&E result was needed for inclusion in the group. In the latter three cases AMI was suspected because recent thrombosis in a coronary artery was known.

Group 2: Eight cases of acute cardiac death (ACD) where gross examination and histology showed the absence of any necrosis but four cases exceptionally showed the presence of a few disseminated necrotic cells using H&E, Luxol or Lie staining. All cases in this group showed advanced stages of stenosing coronary atherosclerosis either in isolation or in combination with recent thrombosis (Table 1 b). Intoxication was excluded in all cases by toxicological analysis.

Group 3: Twelve cases of acute exogenic hypoxia (AEH) due to CO poisoning or hanging.

In the first group serial sections were taken from two tissue blocks extending from the AMI to obviously undamaged myocardium and in the other groups serial sections were taken from five tissue blocks of the left ventricle (anterior, posterior, septum). In case 28 only two tissue blocks (anterior, posterior) could be obtained.

The optimal conditions for the antibodies used were elaborated in serial titration experiments and step-wise variation of all other parameters (Table 2). Pre-treatment procedures and visualisation steps were carried out according to Ortmann and Brinkmann (1997).

Each slide was examined without prior knowledge of the primary diagnosis and re-evaluated after a few months. The intensity and distribution of the staining reactions were scored semiquantitatively.

Antigens with loss, displacement or depletion (troponins, FABP, CD59, myoglobin and desmin) were graded as 0 = negative, 1 = weak and 2 = strong loss of the reaction, compared to an internal standard of normal myocardium.

The reaction of C5b-9 was graded as 0 = negative, 1 = staining of single cells and 2 = staining of cell groups/areas.

Fibrinogen and fibronectin were graded as 0 = either negative or a few disseminated cells with faint staining, 1 = strongly stained disseminated cells or weakly stained patches, 2 = strong reaction of patches or larger areas.

A comparative evaluation showed the duration of fixation had no influence on the strength of the reactivity for C5b-9, fibrinogen and fibronectin.

Results

AMI

All cases had areas of myocardium which showed no abnormalities with the H&E, Luxol and Lie staining methods and nearly all reactions were strongly positive (Table 1 a). The parameters shown (age, sex, post-mortem interval, onset of symptoms) do not seem to be of significance. The subendocardial zone generally escaped necrosis. Only case 1 exhibited incipient infiltration of leucocytes in the necrotic myocardium (Fig. 1 a). Aggregation of C5b-9 could be detected in all cases (Fig. 1 b), but in two cases (cases 2 and 3) only single cells were positive for C5b-9 but were simultaneously negative for the loss of CD59. The degree of depletion for myoglobin, FABP, troponin T and C was of equal intensity and these five antigens also showed correlation in the size of the area affected (Fig. 1 a-e). Also fibrinogen and fibronectin staining was mostly of a greater extent than C5b-9 and corresponded with the depleted areas of the five antigens. The low grading of fibrinogen or desmin did not conform with strong reactivity for C5b-9 in only two cases (cases 7 and 8).

ACD

The reactivity in this group differed widely when compared to group 1. The ACD could have resulted from acute hypoxemia, especially in cases with thrombosis but there also existed one case with acute malignant arrhythmia alone (case 16). The histological finding was consistently contraction band necrosis of single cells and sometimes of groups of cells (cases 9-11) but no coagulation necrosis was detected in routine staining. These three cases (cases 9-11) also exhibited positive C5b-9 deposition whereas CD59 loss was negative but greater areas were depleted for myoglobin, FABP, troponin C and T (Fig. 2 a, b). If the staining for C5b-9 was negative, the immunohistochemical reaction of the other antigens was often weak and the distribution showed more variation (Table 1 b).

Table 2 a–c Details of cases examined and results of the antigen reactions (*CPR* cardiopulmonary resuscitation). **a** Cases of AMI. Case 1 died 216 h after an implantation of a Y-prothesis of the aorta. Other cases demonstrated typical prodromi of AMI. **b** Cases of ACD. Case 16 died suddenly while doing gymnastics exercises; 2 years previously a viral myocarditis had been diagnosed. The post-mortem examination revealed only one microscopic scar in the left ventricle and inconspicuous coronary arteries and conduction system **c** AEH group. Only case 18 exhibited significant coronary arteriosclerosis

a

Case	Sex	Age	Prodromi	Autolysis (h)	Aspect	CPR	FABP	Troponin C	Troponin T	Myoglobin	Desmin	CD59	C5b-9	Fibrinogen	Fibronectin
1	M	76	216 h	20	Yellowish	No	2	2	2	2	2	2	2	2	2
2	F	35	12 h	18	Macroscopic negative	No	2	2	2	2	2	0	1	1	1
3	M	60	7 h	8	Pale	No	2	2	2	2	2	0	1	2	2
4	F	40	6 h	9	Macroscopic negative	No	2	2	2	2	2	2	2	2	2
5	M	77	?	< 96	Pale	No	2	2	2	2	2	2	2	2	2
6	M	60	1 h	20	Pale, hemorrhage	30 min	2	2	2	2	2	2	2	2	2
7	M	39	1 h	85	Macroscopic negative	50 min	2	2	2	2	2	2	2	1	2
8	F	69	0.5 h	39	Pale	No	2	2	2	2	1	2	2	2	2

b

Case	Sex	Age	Prodromi	Autolysis (h)	Etiology	CPR	FABP	Troponin C	Troponin T	Myoglobin	Desmin	CD59	C5b-9	Fibrinogen	Fibronectin
9	F	44	2 h	26	Thrombosis	No	2	2	2	2	1	0	2	2	2
10	F	35	?	< 52	Thrombosis	No	2	2	2	2	2	0	1	2	2
11	M	36	?	< 38	Cardiomyopathy	No	2	2	2	2	2	0	1	1	2
12	F	40	0.5 h	40	Arteriosclerosis	No	2	2	1	2	1	0	0	0	1
13	M	20	0.1 h	18	Arteriosclerosis	30 min	1	1	1	2	1	0	0	1	2
14	F	34	?	< 41	Arteriosclerosis	No	1	1	1	1	2	0	0	0	0
15	M	77	?	< 60	Arteriosclerosis	No	1	1	1	1	0	0	0	1	1
16	M	21	0 h	20	Arrhythmia	60 min	1	1	1	1	1	0	0	0	0

c

Case	Sex	Age	Autolysis (h)	Cause	CPR	FABP	Troponin C	Troponin T	Myoglobin	Desmin	CD59	C5b-9	Fibrinogen	Fibronectin
17	M	14	24	CO 75%	No	1	1	0	1	2	0	0	0	0
18	M	80	24	CO 27%/Arteriosclerosis	No	1	1	0	0	1	0	0	1	0
19	F	12	20	CO 80%	No	1	1	1	1	1	0	0	0	0
20	F	41	26	CO 68%	No	0	0	0	0	1	0	0	1	0
21	M	65	25	Hanging	No	0	0	0	1	0	0	0	0	0
22	M	46	20	Hanging	No	0	0	0	0	0	0	0	1	0
23	F	35	35	Hanging	No	1	1	0	1	0	0	0	1	0
24	F	29	22	Hanging	30 min	1	1	1	1	1	0	0	0	0
25	M	36	24	Hanging	30 min	1	1	1	1	1	0	0	1	1
26	F	29	20	Hanging	30 min	1	1	1	1	0	0	0	1	1
27	M	25	10	Hanging	30 min	1	1	1	1	0	0	0	1	1
28	M	17	18	Hanging, 10 min	80 min	2	2	2	2	2	0	1	2	2

AEH

Histological examination revealed only single cells with contraction band necrosis, in particular in the subendocardial layer in cases of hanging and in case 28, groups of contraction band necrosis. The grading in this group was weak or nearly negative with only few exceptions. Cases of carbon monoxide poisoning and hanging without resuscitation were usually associated with weak reactions for fibrinogen and inconsistently with weak reactions for myocytic proteins. Cases where resuscitation had been attempted showed an increased density of the positive reaction and in one case (case 28) even relatively strong reaction patterns (Fig. 3).

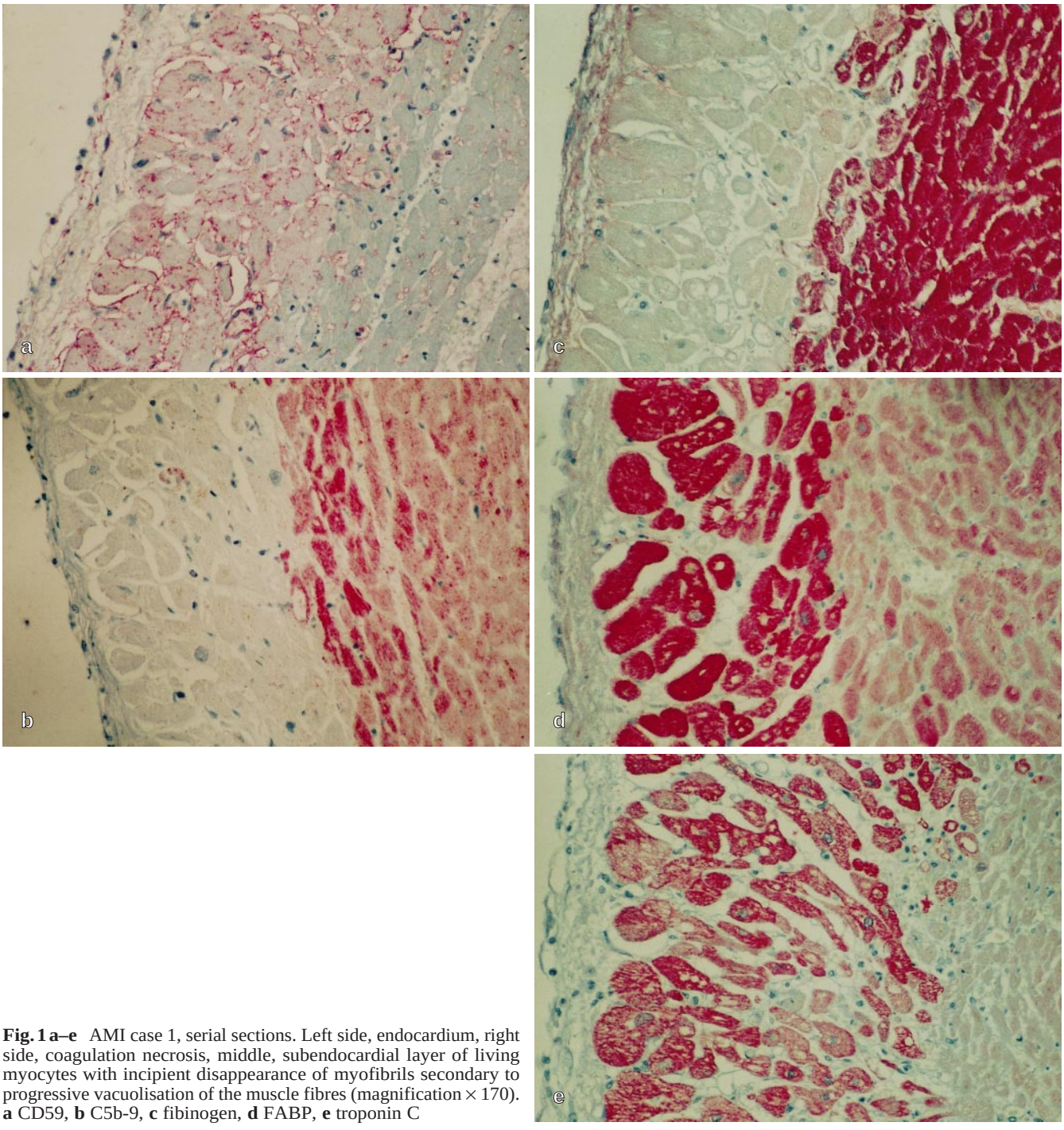

Fig. 1 a–e AMI case 1, serial sections. Left side, endocardium, right side, coagulation necrosis, middle, subendocardial layer of living myocytes with incipient disappearance of myofibrils secondary to progressive vacuolisation of the muscle fibres (magnification × 170). **a** CD59, **b** C5b-9, **c** fibinogen, **d** FABP, **e** troponin C

Discussion

Complement factors

The detection of the complex C5b-9 on and in myocytes becomes positive 30–40 min after the onset of ischemia (Thomsen and Held 1994). In our material the earliest reaction was observed 30 min after the onset of clinical symptoms which would correspond to experimentally induced damages (Hohmeister et al. 1992), although clinical symptoms do not strictly correlate with the beginning of myocardial ischemia.

CD59, also named protectin, inhibits the cytolytic activity of complement by binding to C8 and C9 and thus blocking the formation of the terminal complement complex. The suggestion that this antigen is depleted before C5b-9 deposition occurs (Vakeva et al. 1994) could not be verified in this study. The six cases with mostly single cell C5b-9 deposition showed no loss of CD59. These find-

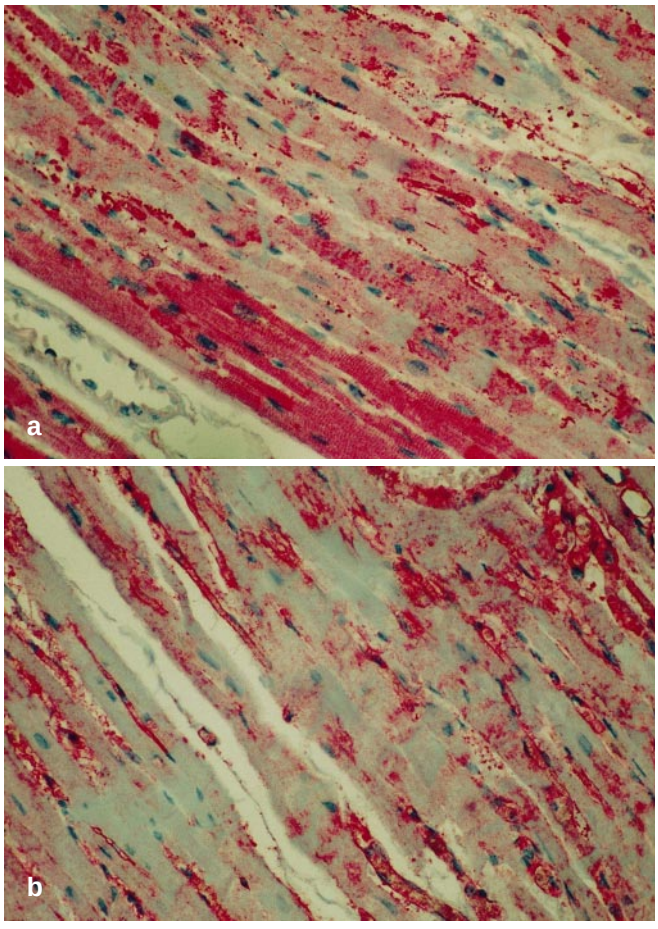

Fig. 2 a, b ACD, case 10. **a** Various stages of depletion of FABP. In a serial section only few cells with contraction band necrosis are positive for C5b-9. **b** A loss of CD59 in cardiomyocytes is also retrospectively not clearly visible. Strong expression of the endothelium

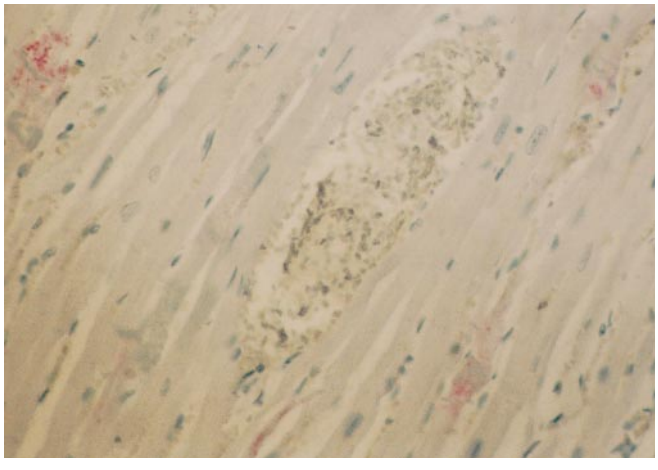

Fig. 3 Case of hanging and prolonged resuscitation attempts (case 28). Positive C5b-9 contraction band necrosis of single cells

ings can be explained if one considers that CD59 reaction is normally very weak in myocytes in contrast to strong expression in the endothelium (Figs. 1 a, 2 b). Thus early release can easily occur from the endothelium without affecting the myocytes.

Myocyte proteins

The immunohistochemical detection of early creatine kinase (CK) depletion in acute heart ischemia was recently published (Amberg 1995). In acute infarctions, the CK plasma level increases after 4–8 h with peak levels after 12-18 h. FABP plasma curves show an even earlier increase with peak levels after 4 h (Kleine et al. 1992). Plasma concentrations of troponin tend to be more specific indicators for AMI. Incipient increases can occur after 3–4 h and peak values are reached in parallel to CK (Bertinchant et al. 1996; Mair et al. 1996).

Depletion of the five markers studied here seemed to start relatively early and to be associated with the course and other changes. All five markers were practically always strongly positive in all cases of group 1. The reaction strength (of depletion!) seemed to correlate with the survival time in group 2 and positive reactions in group 3 were predominantly observed in CPR cases and in carbon monoxide poisoning, although possibly slightly delayed. A comparison between these markers did not offer obvious advantages for the “new” markers compared to myoglobin, but comparison with C5b-9 demonstrated their early reaction. Also, FABP did not seem to be depleted earlier (Table 1), but the high correlation could be used to confirm the depletion area of myoglobin.

These findings indicate a higher sensitivity of the cellular antigens. Troponin T seems to be less effective than troponin C, however the troponin T antibody used was directed against the protein of fast muscle and was therefore a cross-reaction. However, the staining was somewhat paler, which made grading difficult. Apart from this finding, the degree of depletion of the cellular antigens (except desmin) was rather similar. In this investigation myoglobin showed at least a similar or better sensitivity compared with the more recently discovered cellular markers. A higher degree of sensitivity of myoglobin in contrast to desmin has also been described in another study (Brinkmann et al. 1993).

Fibronectin and fibrinogen seem to be deposited intrasarcolemmally early after the beginning of the ischemic process. These markers also become positive earlier than C5b-9 and react in parallel to the cellular antigens. But there existed several cases with positive reactions of the antigens and negative reactions of fibrinogen/fibronectin. Also the areas of depletion of the cellular antigens were usually greater compared to fibrinogen/fibronectin deposition. We therefore concluded that fibrinogen and fibronectin start to become positive later than the cellular antigens but earlier than C5b-9.

Resuscitation attempts (CPR) have an influence on the myocardial integrity (Karch 1987) and two cases with prolonged CPR and myoglobin depletion were recently reported (Leadbetter et al. 1989). Not only does traumatic and electrical damage result in depletion of antigens but

also reperfusion seems to be a potent factor, at least for the deposition of fibronectin and prolonged periods for C5b-9. A grading of immunohistochemistry, the topography of the reaction patterns and histological changes (frequently subendocardial regions) as well as clinical data can avoid misinterpretation in cases of CPR.

The following conclusions can be drawn:

1. The detectable depletion of cellular antigen begins earlier (or visualises weaker damage) than deposition of serum antigens. Weak depletions of cellular cardiac antigens could be detected within minutes after the onset of periods of hypoxia.

2. The deposition of plasma antigens begins earlier (or visualised weaker damage) than that of C5b-9. The terminal complement complex can be visualised 30 min after onset of symptoms of AMI.

3. In the immunohistochemical detection of early myocardial damage myoglobin is at least of the same rank or better than FABP and troponin but in relevant cases a selection of markers from all three categories is strongly recommended.

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