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Immunohistochemical study on tissue transglutaminase and copper-zinc superoxide dismutase in human myocardium: its relevance to apoptosis detected by the nick end labelling method

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Abstract The influence of free radicals on apoptosis was studied in the human heart; 45 autopsy cases were examined by the nick end labelling method (NELM) that detects DNA fragmentation. Immunostaining for copper-zinc superoxide dismutase (CuZn-SOD) and tissue transglutaminase (tTG) induced frequently during apoptosis were also studied. Positive immunoreaction for tTG was detected in mucinous degeneration of myocardial cells; these same cells were also positive for CuZn-SOD but negative for NELM. Myocardial cells showing basophilic alterations after haematoxylin and eosin staining were also positive for CuZn-SOD but negative for the other markers examined. Positive nuclear reaction by NELM was only observed in myocardial cells showing contraction band necrosis or irregularly shaped nuclei surrounding recent or long-standing infarcted foci. In these the other two markers were negative. Since mucinous degeneration lacks the distinguishing morphological features of apoptosis, immunoreactive tTG in this lesion may not imply that the cells are undergoing apoptosis. tTG can be induced in non-apoptotic conditions and may not be involved in apoptosis induced by infarction. Histological disassociation between CuZn-SOD expression and apoptosis suggests the possibility of a cytoprotective role played by endogenous CuZn-SOD against free radical generation in the human heart.

Key words Tissue transglutaminase · Copper-zine superoxide dismutase · Apoptosis · Myocardium

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Introduction

Tissue transglutaminase (EC 2.3.2.13; tTG), an intracellular enzyme, belongs to a family of enzymes that catalyse calcium ion-dependent reactions establishing e(γ-glutamyl)lysine cross-linkings and/or covalent incorporation of di- and polyamines into proteins [3, 9]. In 1987, Fesus et al. [7] indicated that tTG is involved in lead nitrate-induced apoptosis of rat liver and glucocorticoid-induced apoptosis of rat thymocytes. Immunohistochemical study showed that tTG is constitutively expressed in mesangial cells, endothelial cells, smooth muscle cells and chondrocytes [1, 30]. However, many cells express tTG only after the induction of apoptosis [4, 18, 21, 22]. These reports suggest that tTG is not an absolute but an important marker of apoptosis.

In 1992 Gavrieli et al. [10] proposed a nick end labelling method (NELM) to visualize nuclei with internucleosomal cleavage of double strands of DNA (so-called DNA fragmentation) in conventional paraffin-embedded tissue sections at the single-cell level. Their original protocol utilized biotin-labelled dUTP in terminal deoxynucleotidyl transferase (TdT)-induced specific labelling of DNA cleavage, which is detected by peroxidase-labelled avidin [10]. At present, digoxigenin-labelled dUTP is used commonly for detection of apoptosis in situ (ApopTag, Oncor, Gaithersburg, Md., USA). NELM is one of the best ways to detect apoptosis in tissue sections, since DNA fragmentation is characteristically associated with the initiation of the apoptotic process [2, 33].

Recently, Itoh et al. [17] reported that DNA fragmentation occurs in damaged myocardial cells with coagulation necrosis or contraction band necrosis at the margins of acute foci of infarction. This report indicates the possibility that myocardial cells may exhibit DNA fragmentation under adverse pathological events including ischaemia or hypoxia.

DeWall et al. [5] showed that allopurinol provides substantial protection against myocardial ischaemic damage through inhibition of xanthine oxidase generated superoxide-free radicals. Many investigators have since

focused on free radical production and its effect on the heart. In our previous study copper-zinc superoxide dismutase (CuZn-SOD), a cytoplasmic enzyme catalysing the dismutation of superoxide, was identified immunohistochemically in human myocardial cells fixed in calcium-containing formalin [24]. CuZn-SOD-positive myocardial cells were identically positive for luxol fast blue and von Kossa staining [24]. These positive stainings are not identified in tissue sections fixed with formalin without calcium. We suspected that the positive cells were affected by free radical production, and that subsequently, insoluble phospholipids reacting with calcium ions in the fixative were generated and accumulated in the sarcoplasm. We designated this lesion as basophilic alteration (only found in the tissue sections fixed with calcium-containing formalin), because of its purple colour after staining with haematoxylin and eosin (H&E) [24]. Although free radicals are known to be one of the important factors associated with apoptosis in several types of cultured cells [13, 16, 28, 32], their role in induction of apoptosis in the human heart has not yet been clarified.

Constitutive expression of tTG in autopsied adult hearts has been sought but has not been clearly identified [30]. In a preliminary study, we observed that immunoreactive tTG is localized to myocardial cells showing mucinous degeneration. We have examined a larger number of cases and have also extended the study to include another lesion; basophilic alteration. The purpose of the present study was to examine the relationship between apoptosis and the influence of free radicals in human autopsied myocardium in these lesions using NELM and immunostaining for tTG and CuZn-SOD.

Materials and methods

Myocardium was obtained from 45 cases autopsied at Oita Medical University Hospital. There were 36 men and 9 women, ranging in age from 11 to 85 years. Three cases of acute myocardial infarction as a cause of death and 15 cases of interstitial myocardial fibrosis were included, but the others were normal as determined by histological examination. Electrocardiographic abnormalities were found in 21 cases, but there were no cases of hypothyroidism. A transmural tissue slice of 3–5 mm in thickness, taken from the left ventricular anterior wall at the mid-ventricular level, was fixed in 4% buffered formalin in 2% calcium acetate for 5 h at room temperature and embedded in paraffin as in our previous immunohistochemical study on CuZn-SOD in the heart [24]. Five serial 4 μm sections were cut in each case, deparaffinized in xylene, rehydrated in a descending graded series of ethanol. Individual slides were treated as follows: H&E, periodic acid-Schiff (PAS) staining, immunostaining for CuZn-SOD and tTG and the NELM technique employing biotin-labelled dUTP. Because mucinous degeneration is PAS-positive [23, 25], PAS staining was employed for its identification. An additional section from each case was cut and used for NELM employing digoxigenin-labelled dUTP.

For immunostaining for CuZn-SOD, the specimens were predigested in 0.1% protease for 3 min at room temperature, and stained by the streptavidin-biotin (SAB) method (SAB kit, Nichirei, Tokyo, Japan) [27] with anti-CuZn-SOD rabbit antibody produced with purified human CuZn-SOD (Sigma, St. Louis, Mo., USA), whose specificity had been confirmed previously by immunoblotting [24]. The specimens for tTG immunohistochemistry were autoclaved in 0.01 M citric buffer, pH 6.0 at 121°C for 10 min, and immunostained by the SAB method with anti-tTG rabbit serum, which was raised by immunization of synthesized COOH-terminal dodecapeptide (VKGFRNVIIGPA) of human tTG; its specificity had been proven by immunoblotting against guinea pig liver tTG [18]. The reaction products in both immunostainings were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. The control sections stained with phosphate buffered saline (PBS), pH 7.4 or normal rabbit IgG instead of the primary antibody showed negative results.

DNA nick end labelling was performed by the method of Gavrieli et al. [10] with minor modifications. The specimens for NELM were pretreated with 15 μg/ml proteinase K (GIBCO, Gaithersburg, Md., USA) in 10 mM TRIS-hydrochloric acid HCl buffer, pH 8.0 with 1 mM EDTA for 15 min at 22°C, and immersed in TdT buffer, pH 7.2 composed of 30 mM Trizma base, 140 mM sodium cacodylate and 1 mM cobalt chloride for 10 min. The reaction solutions were then applied to the specimens and incubated in a humidified atmosphere at 37°C for 60 min. Subsequently, all specimens were incubated in 300 mM sodium chloride and 30 mM sodium citrate for 20 min to stop the reaction and washed in PBS three times, 5 min each. The signals for end-labelling were visualized using peroxidase-labelled streptavidin (Nichirei) followed by DAB with hydrogen peroxide. The reaction solution contained 300 IU TdT (GIBCO) and biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) in TdT buffer. For the purpose of comparing the sensitivity of NELM by using biotin-labelled dUTP and digoxigenin-labelling, the reaction solution with digoxigenin-11-dUTP was used instead of biotin-16-dUTP and the signals were detected by peroxidase-labelled anti-digoxigenin monoclonal Fab' antibody (Boehringer Mannheim) followed by DAB with hydrogen peroxide. Surgically resected adenocarcinoma of the large intestine processed by procedures identical to those used for the heart was used as a positive control in NELM. Negative control sections, processed without labelled-dUTP, TdT, labelled-streptavidin or labelled-anti-digoxigenin antibody, showed negative results.

The results of immunostaining and NELM (positive or negative) were compared with each other and with other background factors (sex, electrocardiographic abnormality, sepsis, malignant tumour, heart disease or sclerosis of coronary arteries) by Fisher's exact probability test. The relationship between the histological results and the age of the cases or autopsy time after death was also evaluated by the *t*-test.

Results

Mucinous degeneration in the cytoplasm of myocardial cells (Fig. 1a) stained by a positive PAS reaction (Fig. 1b) was found in 36 of 45 cases with different frequencies from one case to another. The distribution of mucinous degeneration was not correlated with that of interstitial fibrosis or acute infarction. All foci of mucinous degeneration examined were positively stained by both anti-CuZn-SOD (Fig. 1c) and anti-tTG immunostaining (Fig. 1d). Both immunoreaction deposits were localized within an amorphous area in the cytoplasm of myocardial cells, which coincided with the area that stained positively by PAS staining. NELM showed negative results in myocardial cells neighbouring mucinous degeneration (Fig. 1e).

Basophilic alteration of the myocardial cytoplasm was identified by H&E staining (Fig. 2a) in 24 cases with a variable frequency from one case to another. The distribution of basophilic alteration was not correlated with that of mucinous degeneration, interstitial fibrosis

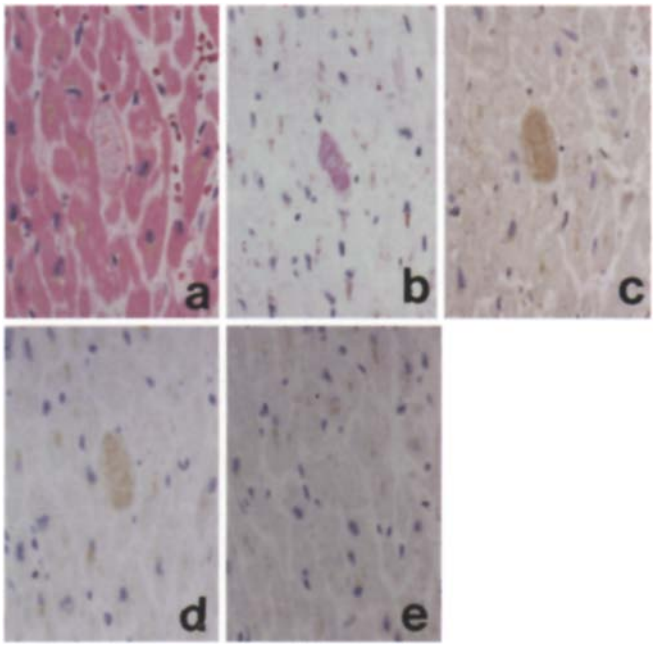


Fig. 1a–e Mucinous degeneration of the myocardium. An area of mucinous degeneration is stained positively by periodic acid Schiff **b**, immunostaining with anti-copper-zinc superoxide dismutase (CuZn-SOD; **c**) and anti-tissue transglutaminase (tTG; **d**) antibodies, but is negative by the nick end labelling method (NELM; **e**). **a–e**, serial sections; **a** haematoxylin and eosin (H&E)

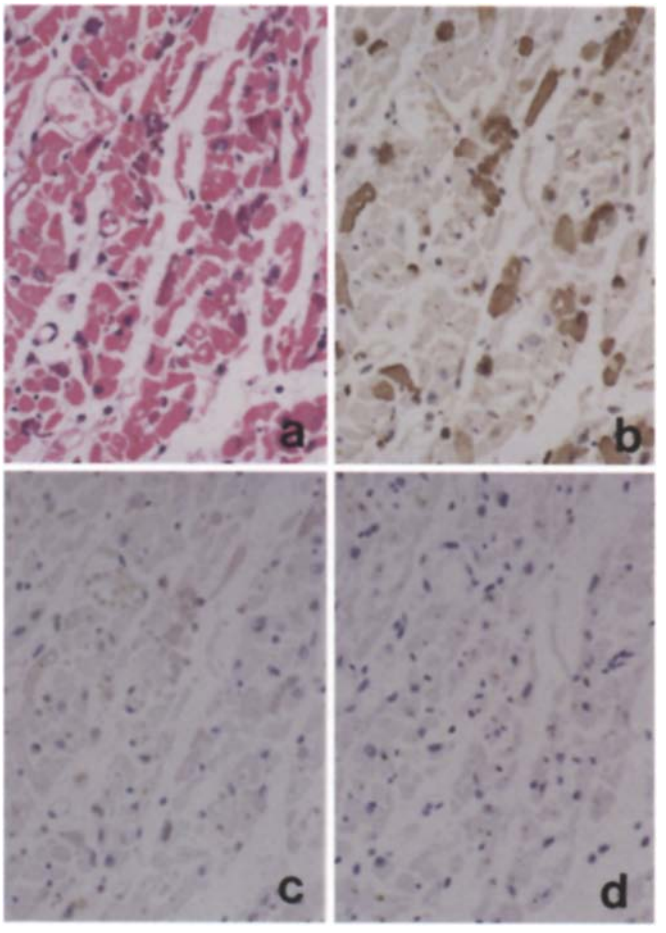
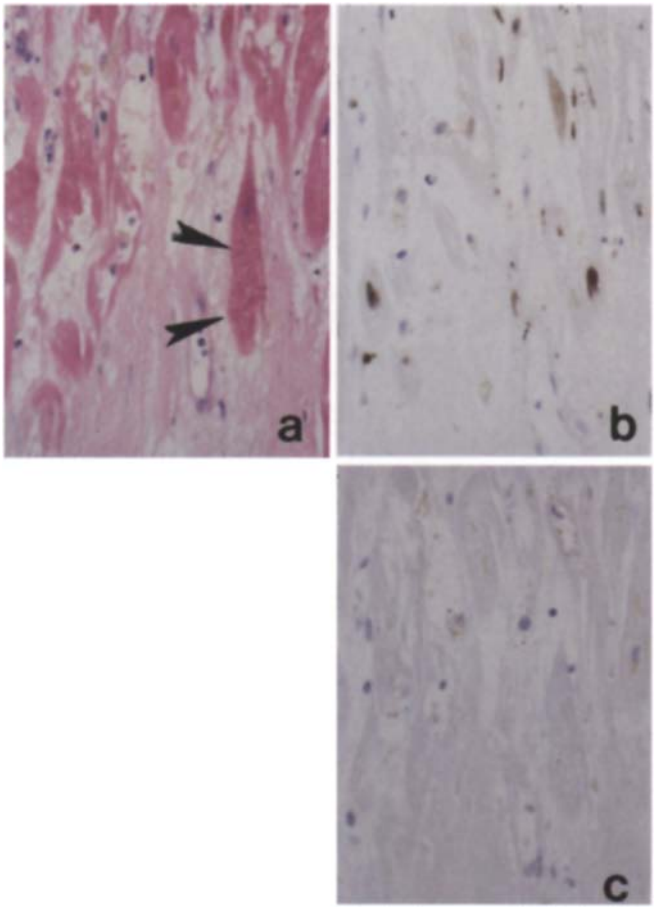


Fig. 2a–d Myocardial cells showing basophilic alteration by H&E staining (**a**) are also positive for anti-CuZn-SOD immunostaining (**b**) but negative for both anti-tTG immunostaining (**c**) and NELM (**d**). **a–d** serial sections



or acute infarction. PAS staining in the area with basophilic alteration was not uniform; some areas were weakly positive and others were negative. Immunostaining for CuZn-SOD in these cells was positive throughout (Fig. 2b), but that for tTG was negative (Fig. 2c). Nuclei of the myocardial cells with basophilic alteration showed no positive reaction by NELM (Fig. 2d).

The NELM-biotin technique exhibited positive nuclear reaction in 12 hearts. The positive myocardial cells were morphologically classified into three groups; myocardial cells with contraction band necrosis surrounding a focus of acute or old infarction (Fig. 3a, b), those with an enlarged and slightly irregularly shaped nucleus and cytoplasm strongly stained with eosin, and those with less morphological change. The immunostaining for CuZn-SOD and tTG (Fig. 3c) was negative in all groups of the NELM-positive cells. By the NELM-digoxigenin technique, however, most of the nuclei of myocardial cells, regardless of the morphological features of the cells, showed a positive reaction. In the positive control (sections of colon cancer) both the frequency and intensity of the reaction were stronger by the digoxigenin

Table 1 Results for immunostaining and the biotin-employed nick end labelling method (*NELM*) in myocardial cells (*PAS* periodic acid-Schiff, *CuZn-SOD* copper-zinc superoxide dismutase, *tTG* tissue transglutaminase, ++ strongly positive, + positive, +/– weakly positive, – negative)

Lesion	PAS	CuZn-SOD	tTG	NELM
Mucinous degeneration	++	+	+	–
Basophilic alteration	+/– or –	++	–	–
Contraction band necrosis or irregularly shaped cells	–	–	–	++
Other morphologically normal cells	–	–	–	+ or –

Table 2 Age of patients and time after death before autopsy (All data are expressed as mean±SEM. There are no significant differences between the groups of "present cases" and "absent ones" by the *t*-test)

	Mucinous degeneration (tTG +, CuZn-SOD +)		Basophilic alteration (tTG –, CuZn-SOD +)		NELM-positive cells[a]	
	present (*n*=36)	absent (*n*=9)	present (*n*=24)	absent (*n*=21)	present (*n*=12)	absent (*n*=33)
Age (years)	64.4±1.9	54.7±6.0	61.5±3.0	63.5±2.6	59.3±5.1	63.6±2.1
Time after death (min)	167.4±20.3	248.8±75.5	173.9±27.7	194.9±36.2	268.3±69.4	152.9±14.6

[a] Positive group by the NELM includes three positive cases of morphologically normal myocardial cells

Table 3 Age of patients and degree of mucinous degeneration (++ more than ten cells in the section showed mucinous degeneration, + two to nine cells, +/– only one cell, – no cells)

Age (years)	Number of cases	Degree of mucinous degeneration			
		–	+/–	+	++
10-19	1	1	0	0	0
20-29	0	0	0	0	0
30-39	2	0	2	0	0
40-49	2	1	1	0	0
50-59	10	2	3	4	1
60-69	17	4	7	5	1
70-79	10	1	2	6	1
80-89	3	0	3	0	0

Table 4 Time after death before autopsy and positivity by the NELM-biotin technique (+ one positive case in morphologically abnormal myocardial cells, – negative, * positive in morphologically normal myocardial cells)

Autopsy time after death (min)	Cases
1-60	+ –
61-120	+ + + + + – – – – – – – – – – – – – – – –
121-180	– – – – – – – – –
181-240	– –
241-300	+ + + – – –
301-360	
361-420	– –
421-480	
481-540	
541-600	
601-660	**
661-720	*

than by the biotin method. In the preliminary study we examined different concentrations (5, 10, 15 and 20 μg/ml) of proteinase K in NELM using biotin-labelled dUTP and found that 15 μg/ml is optimal for detecting the signal in hearts from autopsies. Using other concentrations of proteinase K, digoxigenin-labelled dUTP showed inappropriate results for NELM. The results of immunostaining and NELM are summarized in Table 1.

The average age of the cases with mucinous degeneration was greater than that without it, but this was not significant statistically (Table 2). According to a four point rating scale: ++, more than ten cells in the section showed change; +, two to nine cells; +/-, only one cell; -, no cell showed change, the frequency of mucinous degeneration had a tendency to increase in older individuals, especially in those more than 50 years of age (Table 3). The average time after death before autopsy of the cases with positive results in NELM was greater than those with negative findings, but this finding itself was also not significant (Table 2). In the histogram of time after death before autopsy, NELM-positive cases with contraction band necrosis or nuclear irregularity were distributed in the same range as that of NELM-negative cases (Table 4). However, all three NELM-positive cases without morphological change had been autopsied more than 10 h after death (Table 4). Considering the fact that most of the myocardial nuclei were positive by the NELM-digoxigenin technique and that digoxigenin is more sensitive than the biotin method, this result may be as a result of degradation of DNA producing 3′-OH ends which begins just after death. The positive result in morphologically normal nuclei by the NELM-biotin method may also be produced by detectable degraded DNA accumulated for some time and we concluded that the morphologically normal cells with a positive reaction by

NELM-biotin in the present study can be regarded as a false positive. Thus, the NELM-biotin technique is suitable for autopsied hearts in preference to the digoxigenin approach.

Discussion

Intracytoplasmic degenerative lesion of the myocardium was first described by Geipel in 1905 as a peculiar form of degenerative lesion staining purple with H&E [11]. Since that time this lesion has been variously called "basophile (mukoide) degeneration" [6], basophilic degeneration [14], mucinous degeneration [25] or cardiac colloid [15]. Scotti [25] pointed out that the substance in some of these lesions does not show a basophilic quality. As in our study, most of the lesions were not deeply basophilic but slightly basophilic or amphophilic by H&E staining. To avoid confusion with basophilic alteration, we have called this lesion mucinous degeneration. Although various attempts have been made to identify a causative background disease or pathological state, its pathogenesis and potential causative cardiac or somatic disease are unknown [25]. The heart of patients in the first decade of life rarely shows mucinous degeneration, and the frequency of the lesions is reported to be high in individuals older than 40 years of age [23, 25]. In the present study this tendency was also observed, and there was no significant relationship between the lesion and clinicopathological background factors insofar as we can determine from a review of the clinical histories. The aetiology of mucinous degeneration appears to be associated mainly with aging rather than with specific clinical symptoms.

The biological role of tTG has been widely discussed. Biochemical investigations have shown that tTG-catalysed protein cross-links are irreversible [8] and the insoluble cell envelopes induced are thought to prevent the release of intracellular elements during apoptosis. In several pathological states including experimental models of apoptosis, tTG expression correlates with the onset of apoptosis [4, 21, 22]. However, it is generally agreed that putative apoptotic gene products have other biological roles not concerned with cell death.

According to an ultrastructural study [23], mucinous degeneration, composed of homogeneous material of low electron density, was located in the centre of the myocardial fibre, compressing the nucleus. In contrast, the sequential morphological changes of apoptosis are characterized by chromatin aggregation, concomitant with cytoplasmic condensation in an early stage, followed by nuclear fragmentation. This results in the appearance of apoptotic bodies [33]. We have shown here that NELM could not detect DNA fragmentation around the mucinous lesion and we conclude that myocardial cells with mucinous degeneration are not undergoing apoptosis. Although the biological role of tTG in mucinous degeneration is still unclear, it might contribute to its morphogenesis through the production of irreversible protein cross-links.

Free radicals can induce apoptosis in certain types of cultured cells, and CuZn-SOD acts as an inhibitor for the induction of apoptosis [13, 16, 28, 32]. Since free radicals or lipid peroxide in tissue sections are difficult to visualize at present, immunostaining for CuZn-SOD is one of the best markers for indicating the dynamics of free radicals in tissue sections. Watanabe et al. [31] reported that an intense expression of immunoreactive CuZn-SOD is induced in an experimentally established myonephropathic metabolic syndrome, and suspect that it is caused by free radicals produced in extracardiac regions. It was also reported that the frequency of immunoreactivity for CuZn-SOD in the goblet cells of canine intestine is drastically changed by reperfusion injury, and this change was suppressed by allopurinol [20]. Therefore, we applied immunoreactive CuZn-SOD as a marker that reflects free radical production or its effects.

Myocardial infarction is classically believed to induce cell necrosis through ischaemia or hypoxia. However, recent studies have demonstrated that hypoxia can induce apoptosis in cultured rat cardiomyocytes [29], and myocardial cells of dogs with chronic heart failure experimentally established by intracoronary embolization exhibit apoptosis, especially in the region bordering old infarcts [26]. Kajstura et al.[19] reported that apoptosis is the major form of myocardial damage produced by occlusion of a coronary artery in rats, and that necrosis is apoptotic in type. The process contributes to the progressive loss of cells with time after infarction. It was also reported that human myocardial cells at the margin of acute infarction undergo apoptosis [17], confirmed by the NELM results described here. Although the finding by Itoh et al. [17] that morphologically necrotic cells also show DNA strand breaks seems to be paradoxical, a transient state between apoptosis and necrosis in the marginal area of the infarction may be one of the reasons for the apparent contradiction. Interestingly, Gottlieb et al. [12] showed that apoptosis occurs in the rabbit heart with ischaemia and reperfusion injury, where free radicals are generated progressively, but not in the ischaemic state alone. They indicate that the apoptosis occurring in myocardial cells bordering areas of infarction is possibly mediated through the effects of free radicals. In the present study, however, CuZn-SOD was not found in NELM-positive cells bordering infarction but the enzyme was seen in basophilic alteration that was NELM-negative and not related to infarcted foci. One explanation is that CuZn-SOD expression is less responsive to free radical production. A second possibility is that CuZn-SOD expression is diminished; although CuZn-SOD expression in basophilic alteration may occur immediately before death, NELM-positive cells may be found at a longer time interval after the injury. We suggest that the value of CuZn-SOD expression in studying the pathogenesis of injury in the myocardium should be verified in an experimental model where a time-course response to ischaemia and reperfusion injury could be examined. A third possibility is an increased vulnerability of myocardial cells. In contrast to basophilic alteration, where CuZn-SOD in-

duction is thought to be caused by free radicals arising from an extracardiac region [24, 31], the free radicals affecting NELM-positive cells are believed to be derived locally. The biological function of myocardial cells next to the area of infarction is most likely severely affected by ischaemia or hypoxia, and may not respond with a sufficient amount of CuZn-SOD to act as an effective free radical scavenger. As CuZn-SOD acts cytoprotectively against apoptosis [13], this difference in expression might determine the fate of the injured cell.

Our results indicate that tTG expression in human myocardial cells is not associated with apoptosis induced by recent or long-standing infarction but rather with mucinous degeneration, in which CuZn-SOD is expressed. In addition, myocardial cells with basophilic alteration, which are positive for CuZn-SOD, were shown not to be associated with apoptosis. The possibility that myocardial apoptosis can occur in infarction, without induction of CuZn-SOD may contribute to an understanding of the diversity of the relationship between apoptosis and free radical production in the human heart.

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