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Title

Histopathological characteristics of human cardiac tissues in accidental hypothermia using immunohistochemistry: Immunohistochemical detections of cold, hypoxia and apoptotic related antigens are practicable in the myocardium from hypothermic death even in postmortem examination

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Abstract

We examined the expression of cold-inducible RNA binding proteins (CIRP), RNA binding motif protein 3 (RBM3) hypoxia induced factor 1 (HIF 1) and apoptotic related antigens in the cardiac tissues obtained from hypothermic, coldwater immersion, traffic accidental and cardiac infarction death cases. CIRP, RBM3 and Sirtuin 1 (SIRT1) were expressed in the nucleus of the myocardium obtained from hypothermic and coldwater immersion death cases. Although CIRP and RBPM3 were also expressed in nucleus of the cardiac cells in non-infarcted myocardium obtained from cardiac infarction death cases, SIRT 1 was not detected in the nucleus of the cells both in infarcted and non-infarcted myocardium. The staining results by CIRP RBM3 and SIRT1 may be useful for diagnosis of hypothermic and cold immersion death. Antibodies used in this study, CIRP, RBM 3, SIRT 1, HIF 1, apoptosis inducing factor 1 (AIF 1), vascular endothelial growth factor (VEGF), vascular endothelial growth factor (VEGF), Cathepsin B and D, and protein 53 (p53), stained the cytosol in the cells which contained contraction band and around the contraction band cells, but not contraction band itself. We propose a hypothesis concerning to process of changing in the cardiac tissue affected by cold stress, based in the results obtained from conventional and immunohistochemical staining.

Key Words: Hypothermia, Cardiac cells, Immunohistochemical stain, CIRP, RBPM3, SIRT 1, p-53, Cathepsin B and D, AIFM1, HIF-1, VEGF

Introduction

Hypothermia commonly results from an injury in a cold environment, immersion in cold water or a prolonged exposure to low temperatures without adequate protective clothing and equipment (1). Without thorough review of circumstance, the diagnosis of environmentally induced hypothermia is difficult of affirm at forensic autopsy.

Hypothermia affects the cardiovascular, hematological, neurological, respiratory, renal, metabolic, and gastrointestinal systems (2).

In this examination we studied the expression of cold induced, hypoxia induced and apoptotic related signal pathway proteins in the cardiac tissues from hypothermic and coldwater immersion death.

Materials and Methods

We selected hypothermia related death, coldwater immersion death, traffic accidental death with or without cardiac injury and coronary infarction death cases autopsied at our department during the last four years.

For immunohistochemical staining we used antibodies against CIRP (Protein Tech Group,Chicago, USA), RBM3 (Protein Tech Group,Chicago, USA), Cathepsin B and D (Epitmics, CA,USA), SIRT1 (Novus Biologicals, Litteton,USA), p53 (Santa Cruz, CA, USA), (HIF-1 (Santa cruz CA, USA), VEGF (LSBio. WA, USA) and AIFM1 (LSBio. WA, USA). Immunohistochemical staining was performed by using Histofin SAB-PO kit, and antigen enhancement procedures for each antigen.

Assessment of the staining results was carried out with the Nikon microscope system and pictures were edited in JPEG format.

Results

Immunohistichemical staining

Antibodies against CIRP, RBM3 and SIRT1 showed the reactivity with the nucleus in the cardiac tissues obtained from hypothermic death as shown in Figure 1. Anti CIRP antibody stained the nucleus in the cardiac cells and showed no reactivity with nucleus in epithelial cells of blood vessel in the cardiac tissues from hypothermic death and immersion-drowning victims. Staining intensity was remarkable in hypothermic group compared with immersion-drowning group. This antibody also showed weak reactivity in cytoplasm of cardiac cells containing contraction band from hypothermia and immersion-drowning group, however remarkable reactivity with unknown cells, just like the marcophage, existing in damaged cardiac tissues from cardiac infarction group, and no or feeble reactivity between the antibody and nucleus and/or cytoplasm from traffic accident victims.

Anti RBM3 antibody stained the nucleus in the cardiac cells and showed no reactivity with nucleus in epithelial cells of blood vessel in the cardiac tissues from hypothermic death and immersion-drowning victims. This antibody showed no reactivity with the nucleus in the cardiac tissues from individuals

who died due to traffic accident, and cardiac infarction deaths. Intensity of the reactivity by anti RBM3 with nucleus was stronger than those by CIRP, however no reactivity with cells just like macrophage.

Anti-SIRT1 antibody stained the nucleus in the cardiac tissues from individuals who died from hypothermia and coldwater immersion, but no or feeble with damaged cardiac cells from individuals of traffic accidental and cardiac infarction deaths. This antibody intensively reacted with cytosol of the cells containing contraction bands detected in cardiac tissues from hypothermic related death cases, however no reactivity with contraction band its self. In cardiac infarction the damaged cardiac tissues showed no reactivity with anti-SIRT1.

Antibodies examined in this study, such as Cathepsin B and D, p-53, HIF-1, VEGFA, and AIF1 showed mild reactivity with cardiac cytoplasm in the cells containing contraction band and cells existing around the cells containing contraction band, and showed weak reactivity with the cytoplasm of the cells which exist far from the cells containing contraction band. The reactivity with these antibodies was also observed in the cardiac cells from other cause of death cases, when the contraction bands were detected in the tissue sections. The distribution of the reacting with these antibodies with cytosol of the cells containing no contraction band was extensive in the staining by anti p53 and AIFM1. Although the contraction bands itself in the cardiac cells were clearly

stained by HE and Azan staining, the reactivity with these antibodies with contraction band itself could not be recognized (Figure 2. 3).

Discussion

In a previous study we found four remarkable histological characteristics in the cardiac myocardium obtained from hypothermic and cold-water immersion death, 1) The cardiac cells remarkably closed adherence to each other, 2). The number of red or orange colored cardiac cells by HE or Azan stain, respectively, was frequent than that of control. 3). The cardiac cells with severe vacuolar, colliquative myocytolysis, were identified in the papillary and left ventricle muscles. 4) The contraction bands in the cardiac cells were recognized in all section from the septum of the hearts obtained from hypothermic and coldwater immersion death. In this examination we could find several histological changes of the heart of individuals who died due to hypothermia or immersion in cold fresh water, 1). A reactivity of cold inducible RNA binding proteins were recognized in the nucleus of the cardiomyocyte of the individuals who died due to accidental hypothermia or immersion in cold fresh water. 2). Anti SIRT1 antibody also stained the nucleus of the cardiocytes from individuals who died from hypothermia, immersion-drowning. 3). Clear reactivity with apoptosis progressing or preserving antigens were observed in the legions containing of contraction band necrotic cells and orange colored cells by azan staining, which are mainly existing around the cells containing contraction band.

The nucleus in the cardiocytes from hypothermic death and immersion death cases showed clear reactivity with anti-CIRP and RBM3 antibodies which are expressed to prevent the cell damages caused by hypothermic stress.

CIRP has been described as a nuclear protein whose synthesis is induced by mild hypothermic shock in fibroblastic cells and CIRP was expressed within 6 to 24 hours of cold exposure (3,4). To date, the role of CIRP during mild hypothermia remains controversial, since CIRP is expressed in a large variety of tissues and cells from human and murine origins (5) and it's synthesis is enhanced in response to UV irradiation (6) as well as upon hypoxia (7). In this study, the expression of CIRP was observed in both nucleus and cytosol in cardiac cells, however the expression manner was different, one in cold stress situation and other in cardiac infarction. Based on its mostry nuclear localization, one can speculate that CIRP has some role in nuclear events in hypothermic condition. CIRP has been speculated to protect and restore native RNA conformations during stress, and protects against apoptosis by upregulating extracellular signal-regulated kinase (ERK), which in involved in a cell survival pathway (8). RNA-binding motif protein 3 (RBM3) which is one of two cold shock proteins, may also protect cells from death by acting in a manner similar to the X-linked inhibitor of apoptosis (XIAP) (9). Although Northern blot analysis had shown that the tissue distribution of RBM3 is limited to the pancreas, adrenal glamd, placenta and testis, and is not expressed in the heart (10). Fedorov et al (11) reported that the expression of RBM3 in the heart of hibernation black bears. The existence of the cold shock protein such as CIRP and RBM3 in the nucleus and/or

cytosol in the cardiocytes may indicates that hypothermic state has been kept in the victims and anti- CIRP and RBM3 antibodies may become useful tool to diagnose hypothermic death, although it was reported that the RNA-binding protein belongs to a very small group of cold inducible proteins being synthesized in response to either hypothermia or other conditions of mild stress (12).

In this study several kind of apoptotic related antibodies showed good reactivity with the cardiomyocyte containing contraction band and orange colored materials.

Cathepsin B is a prominent lysosomal protease and plays an important role in the apoptosis process (13). Deiss et al (14) presented for the first time that aspartyl protease cathepsin D is also involved in regulation of apoptosis, and cathepsin D-mediated apoptosis can be induced by cytotoxic factors or prevent apoptosis, which was discussed in animal models (15).

In a normal cell p53 playing as a main regulator of apoptosis is inactivated by mdm2, its negative regulator. Upon DNA damage or other stresses, various pathways will lead to the dissociation of the p53 and mdm2 complex. Once activated, p53 will induce a cell cycle arrest to allow either repair and survival of the cell or apoptosis to discard the damaged cell (16).

SIRT1 is localized in nuclei in general and plays a role in a wide variety of process including stress resistance (17), metabolism, differentiation and aging, and also involved in both anti-apoptotic and pro-apoptosis with modulating p53

(18). However, Jin et al (19) reported that cytoplasm-localized SIRT1 enhances apoptosis. In this study the nucleus were clearly stained and the cytosol were intensively stained compared with those by other antibodies, but the contraction bands were not stained by anti SIRT1 antibody.

AIF is encoded by one single gene located on the X chromosome. AIF is ubiquitously expressed, both in normal tissues and in a variety of cancer cell lines. The precursor is synthesized in the cytosol and is imported into mitochondria. The mature AIF protein is normally confined to the mitochondrial inter-membrane space. In a variety of different apoptosis –inducing conditions, AIF trans-locates through the outer mitochondrial membrane to the cytosol and to the nucleus. Extra-mitochondrial AIF induces nuclear chromatin condensation, as well as large scale DNA fragmentation (20).

Hypoxia-inducible factor (HIF) is a transcription factor that regulates fundamental cellular processes in response to changes in oxygen concentration. Although link between HIF and apoptosis is poorly understood, Sendoel et al (21) identified a novel link between hypoxia and programmed cell death, and paradigm for HIF dictating apoptotic cell fate at a distance by antagonizing p53-mediated apoptosis.

VEGF promotes endothelial cell survival and angiogenesis. Gupta et al (22) examined the signal transduction mechanisms mediating the anti-apoptotic effect of VEGF on stress- and starvation-induced apoptosis and they indicated

VEGF prevent ceramide- and starvation induced apoptosis.

The materials detected by apoptotic related antibodies can divide into two groups, that is, apoptotic stimulating and inhibiting group. Cathepsin B and p53 are stimulating group and AIFM1, HIF-1, VEGF and SIRT 1 are inhibitory group (13-20). Cathepsin D has both characteristics (14,15).

The existence of apoptotic related materials indicate that the apoptotic reaction surely occurs in the cardiac tissues in hypothermic death, and apoptotic reaction may be caused by imbalance between ATP product and consumption in the cardiac tissue in the hypothermic situation. The prolonged exhausting of ATP may cause color changing or contraction band in the cardiomyocytes and these changes together with carcium ion (Ca^{2+}) inflicting and decreasing of pH may be related with appearance of J (Osborn)-Wave (23) in the electrocardiogram from accidental hypothermia. Cell death occurs when ATP production fails to meet the energetic maintenance demands of ionic and osmotic equilibrium. When cold-induced accumulation of Na+ continues unabated, the rise in cytosolic Na^{+} will ultimately lead to membrane depolarization, the opening of voltage-dependent Ca chanels, rapid influx of Ca^{2+} and initiation of membrane phospholipid hydrolysis (24). Deleterious increases in cytosolic Ca^{2+} may arise through a cold-induced breakdown of plasma membrane Na/Ca exchange, by an imbalance between rates of ATPase-dependent Ca^{2+} uptake, by the sarcoplasmic reticulum and rates of

Ca^{2+} efflux and/or by pH-dependent activation of Ca^{2+} efflux from the sarcoplasmic reticulum (24). Once initiated, the pathological series of effects leading to necrotic cell death during prolong hypothermia may be largely uncontrollable and analogous to the irreversible membrane injury and dissipation of ion gradients during anoxia (25).

We will propose a hypothesis based on the results obtained from this examination together with previous one. At mild hypothermia the cardiac cell expresses CIRP, RBPM3 and SIRT1 to protect itself (17), and with continuous lowering the body temperature the lacking of ATP occurs in the cells. Decreasing of ATP brings about gain of exciting contraction coupling, and then makes cell adherence, contraction band and eosinophilic or orange color change in cardiac cells. Further decreasing of ATP causes expression of hypoxia inducing factors and contraction band necrosis in the cardiac cells. After that apoptotic preserving factors such as AIFM1, HIF-1, VEGF and SIRT 1 in cytosol of cardiac cells. On the contrally apoptotic stimulating factors such as Cathepsin B and D, and p53 will be expressed and cell death finally occurs with contraction band necrosis or vacuolization.

The expression of cold shock proteins in the cardiac tissue may firstly occur, and then expression of apoptotic related antigens occurs. After that small amount of the cardiac cells changes into hypereosinophilic situation which indicate oncosis of the cells, and the cardiac cells change finally into vacuolated cardiac cells

which indicates necrotic cells, before the individual death by hypothermia.

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Figure Legends

Figure.1 : Anti CIRP (1a) and RBM3 (1b) stained the nucleus and cytosol in the cardiac cells, while anti SIRT1 (1c) stained only nucleus.

Figure.2 : Anti Cathepsin B (2a) and D (2b) and p53 (2c) stained cytosol in the cells containing contraction band but not contraction band itself.

Figure.3 : Anti HIF (3a), AIF (3b)and VEGF (3c) stained cytosol in the cells containing contraction band but not contraction band itself.

What is already known on this topic

- The diagnosis of environmentally induced hypothermia is difficult of affirm at forensic autopsy. We could find several histological changes of the heart of individuals who died due to hypothermia or immersion in cold fresh water.

What This study adds

- We found immunohistochemical findings in the cardiac tissues. There are no report about process of changing in the cardiac tissue affected by cold stress, based in the results obtained from conventional and immunohistochemical staining.

Suggestions for further development

- The expression of CIRP and RBPM3 in the nucleus of the cardiac cells may play important roles for diagnosis of hypothermic death. Apoptosis progressive or protective reactions by antibodies were obviously recognized both in hypothermic death. Both ATP reduction and direct reduction of oxygen might occur in cardiac tissues in hypothermic death.