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Oxidative stress may be involved in distant organ failure in tourniquet shock model mice

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ABSTRACT

Crush syndrome is characterized by prolonged shock resulting from extensive muscle damage and multiple organ failure. However, the pathogenesis of multiple organ failure has not yet been completely elucidated. Therefore, we investigated the molecular biological and histopathological aspects of distant organ injury in crush syndrome by using tourniquet shock model mice. DNA microarray analysis of the soleus muscle showed an increase in the mRNA levels of Cox-2, Hsp70, c-fos, and IL-6, at 3 h after ischemia/reperfusion injury at the lower extremity. *In vivo* staining with hematoxylin and eosin (HE) showed edema and degeneration in the soleus muscle, but no change in the distant organs. Immunohistological staining of the HSP70 protein revealed nuclear translocation in the soleus muscle, kidney, liver, and lung. The c-fos mRNA levels were elevated in the soleus muscle, kidney, and liver, displaying nuclear translocation of c-FOS protein. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis suggested the involvement of apoptosis in ischemia/reperfusion injury in the soleus muscle. Apoptotic cells were not found in greater quantities in the kidney. Oxidative stress, as determined using a free radical elective evaluator (d-ROM test), markedly increased after ischemia/reperfusion injury. Therefore, examination of immunohistological changes and determination of oxidative stress are proposed to be useful in evaluating the extent of tourniquet shock, even before changes are observed by HE staining.

1. Introduction

Crush syndrome is characterized by prolonged shock resulting from extensive muscle damage and multiple organ failure. However, the pathogenesis of multiple organ failure has not yet been completely elucidated. Therefore, the causal relationship between crush muscle injury and death is often controversial in forensic practice. Researchers investigating crush syndrome *in vivo* have often used an animal tourniquet shock model, involving an animal whose limbs have been damaged by ischemia/reperfusion injury (I/R) [1–7]. As shock is a functional reaction of the vascular system to bodily injury, the presence of shock is difficult to prove by morphological examination [8]. Accordingly, the elucidation of pathogenesis and abnormal histopathology of distant organ failure resulting from extensive crush muscle injury will help not only in clinical diagnosis and therapy but also in forensic autopsy diagnosis by functioning as postmortem markers.

Although several studies have reported that apoptosis is implicated in organ injury after I/R of a local organ (the heart, kidney, liver, or skeletal muscle) [9–12], it remains unclear whether apoptosis is involved in the injury of distant organs. A previous study showed that the lipid peroxide concentration was elevated in the skeletal muscle, kidney, and liver during tourniquet shock [5]; however, the level of reactive oxygen metabolites (ROMs) in the blood has not yet been determined.

In this study, we investigated the stress response factors and apoptosis-related factors in the skeletal muscle and distant organs after I/R by using molecular biological and histopathological techniques. We also determined the blood levels of oxidative stress.

2. Materials and methods

2.1. Animal model

All animal experiments were approved by the Animal Research Committee in accordance with the Guidelines for Animal Experiments of Fukushima Medical University School of Medicine and the Japanese Government Animal Protection and Management Law.

C57BL/6 J male mice (11–13 weeks old and weighing 21–30 g) were used in the experiments. Mice were anesthetized with intraperitoneal injection of pentobarbital sodium (80 mg/kg body weight). The animals were divided into two groups: a bilateral I/R group in which a rubber band (No. 8, Kyowa, Osaka, Japan) was wound seven times around the inguinal region of each hind limb

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and kept in place for 3 h followed by reperfusion for 3 h (Group TR3) and a control group in which an anesthetized condition was maintained for 6 h without rubber bands attached to hind limbs (Group C). At the time of sacrifice, the mice were deeply re-anesthetized and blood samples were obtained by direct cardiac puncture with heparin. The liver and both kidneys, lungs and soleus muscles were removed rapidly after transcardiac perfusion with saline.

2.2. DNA microarray

DNA microarray analysis was carried out using samples from soleus muscles of group TR3 ($n = 3$) and group C ($n = 3$). Total RNA extraction, complementary RNA probe generation, hybridization to an Affimetrix mouse genome 430 2.0 array, scanning and analysis were conducted at TAKARA Bio (Shiga, Japan). The microarray contains over 39,000 transcripts representing 34,000 genes.

2.3. Real-time PCR

Gene expression levels in each organ from mice in group TR3 ($n = 12$) and group C ($n = 12$) were determined. Total RNA was isolated from samples using TRIZOL Reagent (Invitrogen, California, USA) according to the manufacturer's instructions. Two micrograms of total RNA was reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, California, USA). Real-time PCR was performed using a Step One® Real-time PCR System (Applied Biosystems, California, USA). The primers used were those for Taqman® Gene Expression Assays (Applied Biosystems, California, USA): Hsp70 (Hspa1b; Assay ID: Mm03038954_s1), c-fos (Mm01307334_g1), Cox-2 (Ptgs2; Mm01302932_g1), Bcl-2 (Mm00477631_m1) and Caspase-3 (Mm01195085_m1). The reaction system was 10 μl of Taqman® Gene Expression Master Mix (Applied Biosystems, California, USA), 1 μl of primers and 7 μl of nuclease-free water, and 2 μl of cDNA was used as the template for application. The PCR program was 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was measured triply and the quantity was determined by the calibration curve method. Hsp70, c-fos, Cox-2, Bcl-2 and Caspase-3 mRNA expression levels are shown as ratios of GAPDH. The fold change for a gene in group TR3 was calculated relative to the level in group C.

2.4. Histology

Parts of the organs ($n = 3$ respectively) were fixed with 4% paraformaldehyde in phosphate buffer, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

For immunohistochemical study, the sections were deparaffinized and washed in water (5 min). After incubation in 3% hydrogen peroxide in PBS (15 min), slides were washed in PBS (5 min × 3 times). For antigen retrieval, slides were heated in 10 mM citrate buffer (95 °C, 10 min) and naturally cooled. After washing in PBS (5 min × 3 times), slides were incubated with 1:100-diluted anti-HSP70 (sc-1060, Santa Cruz Biotechnology, California, USA), anti-c-FOS (sc-253, Santa Cruz Biotechnology, California, USA), anti-COX-2 (CAY-160126, Cayman Chemical Company, Michigan, USA), and anti-Bcl-2 (sc-492, Santa Cruz Biotechnology, California, USA) at 4 °C overnight. An anti-rabbit polyclonal IgG (Histofine Simple Stain Mouse MAX-PO® Nichirei, Tokyo, Japan) was used as the secondary antibody (20 min). After washing in PBS (5 min × 3 times), the diaminobenzidine method (Simple Stain DAB, Nichirei, Tokyo, Japan) was used for visualization (5–20 min). Nuclear staining with hematoxylin, dehydration, clearing, mounting and covering were performed. Within 10 microscopic fields (×400 magnification) selected at random on the sample, stained and non-stained nuclei were counted. Mean ratios of the stained nuclei and standard errors were calculated. Muscle cells in soleus muscles, cortical renal tubular cells in kidneys, hepatocytes in livers and alveolar epithelial cells in lungs were observed.

2.5. TUNEL staining

Sections of soleus muscles and kidneys ($n = 3$ respectively) were stained using TdT-mediated dUTP-biotin nick end labeling (TUNEL). A commercially available kit (Apop Tag® Peroxidase In Situ Apoptosis Detection Kit S7100; CHEMICON, California, USA) was used according to the manufacturer's instructions. Nuclear staining with hematoxylin was done. TUNEL-positive cells within ten microscopic fields (×400 magnification) selected at random on the sample were counted. Mean ratios of TUNEL-positive cells and standard errors were calculated.

2.6. Oxidative stress marker (d-ROM test)

Blood from mice in group C and group TR3 ($n = 8$ respectively) was centrifuged. Blood samples from mice in which bilateral hind limbs had been bound for 3 h (group TR0, $n = 6$) and blood samples from mice in which limbs had been bound for 3 h and reperfused for 1 h (group TR1, $n = 6$) were also used in.

Reactive oxygen metabolites (ROMs) were measured using the Free Radical Elective Evaluator (Diacron International, Italy). Twenty microlitre of plasma was mixed with an acetic acid buffer solution of pH 4.8 to stabilize the hydrogen ion concentration. In an acidified medium, bivalent and trivalent iron from the protein component of the blood ionized and worked as a catalyst to break down hydroperoxides in blood into alkoxyl and peroxy radicals to become free radicals. These were transferred into a cuvette containing colorless chromogen (*N*,*N*,diethylparaphenylenediamine), which is oxidized by free radicals and changes into a radical cation with a magenta color. The density of the magenta color reflects the concentration of hydroxides in blood, which is proportional to the quantity of ROMs. The magenta color was measured using a photometer (505 nm). Since the concentrations of various hydroperoxides in blood were measured, the values are reported in the arbitrary unit U.CARR. One U.CARR is equivalent to 0.08 mg/dl of hydrogen peroxide.

2.7. Statistical analysis

Data for gene expression levels and oxidative stress markers are shown as means ± standard errors. Comparisons among samples were performed using the Mann–Whitney U test. Data for stained nuclear ratios and TUNEL-positive ratios are shown as means ± standard deviation. A Shapiro–Wilk test showed that the samples followed normal distribution; the data was then analyzed for statistical significance with a *t*-test. All data were analyzed using SPSS ver. 17.0 (SPSS Inc., Chicago, USA). The criterion for significance was set at $p < 0.05$.

3. Results

The expression levels of 997 genes in group TR3 were more than twofold higher than those in group C, whereas the expression levels of 808 genes in group TR3 were less than half those in group C. The probe with the most elevated intensity was that of the Cox-2 gene. The second, fourth, and seventh probes were all of the Hsp70 gene. The sixth probe was that of the c-fos gene, an immediate early gene. The tenth probe was that of interleukin-6, an inflammatory cytokine (Supplemental Table 1). The probes of decreased