The caspase inhibitor, Z-Val-Ala-Asp(Ome)-CH$_2$F (ZVAD-fmk), reduces the number of myocytes with DNA fragmentation and reduces the size of the myocardial infarct in rats, but the caspase inhibitors, YVAD and DEVD, did not reduce the infarct size (IS) in rat hearts, although they inhibited caspase activation and reduced the incidence of myocyte DNA fragmentation. Thus, it is controversial whether caspase inhibitors reduce IS. In addition, each caspase inhibitor reduced the number of myocytes with DNA fragmentation to approximately half, depending on the caspase activity, but the remaining 50% may not depend on caspase activity because, in general, DNA fragmentation is induced via both serine protease-dependent and caspase-dependent pathways. The serine protease-dependent DNA fragmentation is suppressed by serine protease inhibitors such as 3,4-dichloroisocoumarin (DCI), but not by caspase inhibitors, so we hypothesized that serine protease DNA fragmentation is also involved in infarcted myocytes and is related to the IS. Therefore, we investigated whether ZVAD-fmk or DCI reduces the incidence of DNA fragmentation and whether these inhibitors reduce the size of the myocardial infarct induced by ischemia–reperfusion.

**Methods**

The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan. All rabbits received humane care in accordance with the ‘Guide for the Care and Use of Laboratory Animals’, published by the U.S. National Institutes of Health (NIH).
publication 8523, revised 1985).

Surgical Preparation

Male Japanese white rabbits, weighing 1.9–2.5 kg, were anesthetized with 30 mg/kg sodium pentobarbital and mechanically ventilated with room air. All surgical procedures were performed aseptically. The left carotid artery and jugular vein were cannulated to monitor arterial blood pressure and to administer drugs or saline, respectively. After a left thoracotomy in the third intercostal space, the heart was exposed and a 4-0 silk suture loop was placed around the large branch of the coronary artery coursing down the middle of the anterolateral surface of the left ventricle (LV). Occlusion and reperfusion were performed by pulling or releasing a snare around the loop. Myocardial ischemia was confirmed by regional cyanosis and electrocardiographic change. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snare.

Estimation of Infarct Size

Rabbits were assigned randomly to 3 groups: control, ZVAD or DCI groups. In the control group (n=8), 1 ml of placebo (dimethylsulfoxide (DMSO)) was injected 20 min before occlusion and 60 min after reperfusion; in the DCI group (n=8), DCI (2 mg/kg, dissolved in DMSO) was intravenously infused 20 min before occlusion; in the ZVAD group (n=8), 0.8 mg/kg iv, dissolved in DMSO) was infused 20 min before occlusion and 60 min after reperfusion. After the experiment, the chest was closed and the rabbits were allowed to recover from anesthesia for 2 days. At 48 h after reperfusion, the rabbits were heparinized (500 U/kg) and killed by an overdose of pentobarbital. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded and monastral blue dye (4%, Sigma Chemical Co, St Louis, MO, USA) was injected from the aorta at 80 mmHg. The LV was sectioned into 7 slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of triphenyl tetrazolium chloride (TTC) at 37°C to visualize the infarct area and photographed. The area of the ischemic region and of the infarcted myocardium were traced on each LV slice and photographed. The area of the ischemic region and of the infarcted myocardium were traced on each LV slice and photographed. The area of the ischemic region and of the infarcted myocardium were traced on each LV slice and photographed. The area of the ischemic region and of the infarcted myocardium were traced on each LV slice and photographed.

Detection of dUTP Nick End-Labeling (TUNEL)-Positive Cells

The surgical procedure was as described. Rabbits underwent 30 min ischemia followed by 4 h reperfusion. In the control group (n=7), 1 ml of placebo (DMSO) was injected 20 min before ischemia. In the DCI group (n=7), each rabbit was intravenously infused with DCI (2 mg/kg) at 20 min before coronary occlusion, and in the ZVAD group (n=7), rabbits were intravenously injected with ZVAD-fmk (0.8 mg/kg) at 20 min before coronary occlusion and at 90 min after reperfusion. The rabbits were killed 4 h after reperfusion and the heart was excised. After fixation in 10% phosphate-buffered formalin for 3 days, the free walls of the atria and the right ventricle were removed. The left ventricle was sectioned into 7 slices parallel to the atrioventricular ring and the fixed transverse slices were embedded in paraffin. After deparaffinization and rehydration, two or three 4-μm thick serial sections were stained with hematoxylin and Masson’s trichrome. The DNA fragments in the third section were determined using an ApopTag in situ apoptosis detection kit (Oncor). The DNA nick was labeled according to the supplier’s instructions, which were based on the method of Schmitz et al. After TUNEL, the sections were counterstained with hematoxylin.

Cardiomyocytes in the infarcted area were counted using a light microscope. In each specimen, cardiomyocytes with counterstained nuclei were counted in 60 random high-power fields (×400) from the endocardial portion of the infarcted areas. Myocytes in which the nucleus was obviously labeled with diaminobenzidine were defined as TUNEL-positive and the percentage of these in approximately 3,000 myocytes with a nucleus in the infarcted area was then calculated. This evaluation was carried out independently by 2 persons who were unaware of the experimental protocol.

Agarose Gel Electrophoresis

In a separate series of experiments, rabbits underwent 30 min ischemia followed by 4 h reperfusion with and without ZVAD or DCI, as in the previous protocol. Rabbits (n=5 in each group) were killed and tissue samples were taken from the center of the risk area of the LV, which was separated by monastral blue dye perfusion, then frozen in liquid nitrogen and stored at −80°C until analysis. The frozen samples were mechanically homogenized on ice, lysed with lysis buffer containing 10% sodium dodecyl sulfate, 10 mmol/L Tris, and 1 mmol/L EDTA (pH 7.8) and digested with protein kinase K at 200 mg/ml at 37°C for 16 h. The DNA was purified by extraction with phenol/chloroform and dissolved in TE buffer (10 mmol/L Tris and 1 mmol/L

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MBP, mean blood pressure; ZVAD, ZVAD-fmk; DCI, 3,4-dichloroisocoumarin. The number of rabbits was 8 in each of the control, ZVAD, and DCI groups. *p<0.05 vs control.
EDTA). The concentration and purity of DNA were determined by the measurement of the optical density at 260 nm and the ratio of optical density at 260 nm to that at 280 nm. DNA (4 mg) was run on 2.0% agarose gel. The DNA was visualized with ethidium bromide.

**Electron Microscopic Findings**

Tissue samples were taken from the center of the risk area of the LV from the rabbits subjected to 30 min ischemia followed by 4 h reperfusion with and without ZVAD or DCI. The samples were cut into 1-mm cubes and fixed for 4 h at 4°C in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. They were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanols, and embedded in epoxy resin. Thin sections (80 nm) were cut with a diamond knife and collected on bare 300-mesh nickel grids. They were stained with uranyl acetate and lead citrate, and examined using an electron microscope (Hitachi 700).

**Statistical Analysis**

Values are expressed as the group mean ± SEM. To compare the group means of area at risk (AAR), IS and the incidence of TUNEL-positive cells, one-way analysis of variance (ANOVA) was performed and if ANOVA was significant, a modified unpaired t test was carried out to assess which group was significantly different. The effects of treatments on hemodynamics were analyzed using one-way ANOVA with repeated measures. Differences with a value of p<0.05 were considered statistically significant.

**Results**

**Mortality and Animal Exclusion**

Thirty-one rabbits were initially enrolled in the IS study. There was no significant difference in the incidence of ventricular fibrillation and mortality. One rabbit in each of the control groups developed ventricular fibrillation during coronary occlusion, and 1 rabbit developed ventricular fibrillation during reperfusion in the each of the ZVAD and DCI groups; all these rabbits died. Thus, the experiments were completed in the remaining 28 rabbits and the findings from these animals were used for the analysis of IS.

**Hemodynamic Parameters (Table I)**

There was no significant difference in heart rate among the 3 groups. Mean blood pressure and double product decreased during ischemia and reperfusion in the ZVAD and DCI groups compared with the controls. ZVAD, ZVAD-fmk; DCI, 3,4-dichloroisocoumarin. *p<0.05 vs control.

**Infarct Size**

The mean percentages of the AAR (% LV) were 26.3±3, 25.6±2.8, 25.6±2 in the control, ZVAD and DCI groups, respectively (Fig 1A). No significant difference was seen and DCI groups compared with the controls.
among the groups. As shown in Fig 1B, the IS as a percent-
age of the AAR was unchanged in the ZVAD (41.3±4.5%, n=8) and DCI (50.4±3.8%, n=8) groups compared with the saline control group (43.5±4.5%, n=8).

DNA Fragmentation Measured by TUNEL

Myocytes with TUNEL-positive nuclei were observed in the infarcted myocytes induced by 30 min of ischemia and 4 h of reperfusion (Fig 2). The percentage of myocytes with DNA fragmentation, which was assessed by TUNEL in the infarcted area, was significantly reduced in the ZVAD (3.5±0.8%, p<0.05) and DCI groups (4.2±0.9%) compared with the control group (10.7±1.9%) (Fig 3).

Agarose Gel Electrophoresis

DNA laddering, indicative of DNA fragmentation, was demonstrated in myocardial specimens sampled from the risk area of the controls, but not in 4 of 5 samples from the ZVAD and DCI groups (Fig 4, Table 2).

Electron Microscopic Findings

We observed cardiomyocytes with severely edematous cytoplasm, grossly swollen mitochondria with many amorphous dense bodies, frequently disrupted cytoplasmic membranes, marked clumps of chromatin material that were of various sizes and randomly dispersed in the nuclei, disappearance of glycogen granules, and formation of numerous contraction bands of myofibers. These ultrastructural changes suggest irreversible oncosis (necrosis) and were seen equally in the control, ZVAD and DCI groups. There was no evidence of apoptotic ultrastructures, such as half-moon or crescent-like condensation of nuclear chromatin, apoptotic bodies etc in any of the 3 groups.

Discussion

The present study demonstrated that treatment with ZVAD and DCI significantly reduced the incidence of TUNEL-positive myocytes in the infarcted area, but did not reduce the myocardial IS.

Caspase-Dependent and Serine Protease-Dependent DNA Fragmentation of Infarcted Myocytes

We previously reported that DNA fragmentation, assessed by the DNA ladder and TUNEL methods, appeared 2–4 h after reperfusion in a rabbit model of 30 min ischemia and reperfusion. In the present study, therefore, we assessed DNA fragmentation by the TUNEL method 4 h after reperfusion in rabbits with 30 min ischemia, and found that TUNEL-positive myocytes appeared in the infarcted area and that treatment with ZVAD and DCI significantly reduced the incidence of TUNEL-positive myocytes and DNA laddering, respectively, in the infarcted area.

Caspase-dependent DNA fragmentation can be inhibited by caspase inhibitors. ZVAD-fmk is a broad and irreversible caspase inhibitor that reduces the incidence of DNA fragmentation. Okamura et al reported that YVAD-aldehyde, a caspase-1-like protease inhibitor, and DEVD-aldehyde, a caspase-3-like protease inhibitor, inhibited caspase activation and approximately 50% of myocyte DNA fragmentation, which we confirmed in the present study. However, the DNA fragmentation associated with the serine protease-dependent pathway is suppressed by serine protease inhibitors, such as DCI, but not by caspase inhibitors, and the present study showed that approximately 50% of the myocyte DNA fragmentation disappeared with DCI treatment. These findings suggest that the serine protease-dependent pathway, as well as the caspase-dependent pathway, is involved in the DNA fragmentation of infarcted myocytes.

We reported previously that the cellular damage of infarcted myocytes proceeds from reversible or irreversible oncosis without DNA fragmentation to irreversible oncosis with or without DNA fragmentation. In that study, there was no evidence of cardiomyocytes with apoptotic ultrastructures, which indicated that DNA fragmentation occurs in the cardiomyocytes that are already irreversibly onotic, but not apoptotic. The findings of the present study confirmed this.

Infarct Size and Caspase or Serine Protease Inhibitor

Yaoita et al reported that ZVAD-fmk reduced the IS as a percentage of the AAR from 66.6±3.7% into 52.4±4.0% in rats, and although the difference was very small, they described it as significant. On the other hand, Okamura et al reported that YVAD-aldehyde and DEVD-aldehyde did not significantly reduce the IS in rats. In the present study, we showed that neither DCI nor ZVAD-fmk reduced the IS in rabbits. In all of these studies, DNA fragmentation, as indicated by TUNEL and DNA laddering, was definitely decreased, which suggests that the doses of the caspase and
serine protease inhibitors were sufficient. ZVAD and DCI decreased the mean blood pressure without any effect on heart rate. It is generally considered that a decrease in the double product as the result of a decrease in the mean blood pressure reflects a reduction in the IS. On the other hand, the incidence of TUNEL-positive myocytes ranged from 11.1±1.0% to 20±1% in these studies, indicating that the role of DNA fragmentation in determining IS was small. That is, even if caspase and serine protease inhibitors can salvage all cardiomyocytes with DNA fragmentation, the infarct-reducing effect is 10–20%.

In conclusion, serine protease-dependent DNA fragmentation does occur in infarcted myocytes, in addition to caspase-dependent DNA fragmentation, but infarct-reducing effect of these inhibitors is very small because of the low incidence of infarcted cardiomyocytes with DNA fragmentation.

Acknowledgment

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References