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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ　TUNEL染色陽性を示すラット急性虚血心筋細胞の細胞膜透過性について
-- TUNEL染色法とLanthanumイオンを用いた研究 --

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表　有 ・ (無)　(学会名・内容)

(2) 学会誌等に発表した論文　(有) ・ 無　(雑誌名・論文名)

Basic and Applied Myology. 9(1):29-34,1999 (Italy)

Rat Cardiomyocytes with TUNEL-Positive Nuclei Induced by Permanent Ischemia Show Increased Plasma-Membrane Permeability Monitored by a Combined Use of TUNEL Method and Lanthanum Ions

3．今後の研究計画

ラット急性虚血心筋細胞の細胞死のメカニズム(apoptosis関連蛋白の調節)について

①ラット左冠状動脈の前下行枝を結紮し、2h, 4h, 6h, 8h, 12h, 1day後に心臓を摘出し、虚血巣を二分割し、ホルマリン固定と4%PFA固定(凍結切片用とEpon包埋・LR white包埋用)をする。

②パラフィン切片のHE染色とTUNEL染色。

③通常電顕でのミトコンドリアの形態学観察。

④凍結切片のcytochrome c, caspase 3, caspase 9, bcl-2, bax等apoptosis関連蛋白の免疫組織化学染色。

⑤金コロ免疫染色法にてcytochrome c, caspase 3, caspase 9の細胞内分布を透過型電子顕微鏡下で観察する。

4．研究指導者の意見

心筋が虚血に陥ると細胞が死ぬ。その際アポトーシスと壊死の両方の機序が働くが、アポトーシスが先行し、壊死に移行するのか、それとも、両者が別々の細胞で独立して発症し、細胞が死滅するのか、はっきりしていなかった。今回の Dr. Tong, Jie (佟杰) の研究により、虚血早期の心筋ではアポトーシス (DNA 断片化) と壊死 (細胞膜透過性亢進) の機序が同一細胞でほぼ同時に進行していることがはっきりした。この研究遂行に当たり、DNA 断片化 (アポトーシスの指標) 細胞の細胞膜透過性亢進 (壊死の指標) を証明する為の方法が独創的、特異的である。即ち、虚血心筋を La を含む固定液で処理し、凍結切片を作成し、TUNEL 染色により DNA 断片化細胞を確認し、更に電顕用に再固定、樹脂包埋、薄切、電顕観察し、La の透過性を検索し、始めて納得のいく結果が得られた。これは彼女の優れた能力によるもので、今まではっきりしなかった虚血初期の心筋細胞の病態をあきらかにすることができた。彼女の研究者としての能力を高く評価したい。

研究指導者氏名 伊藤元 印

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい (枚数自由・ワープロ使用)

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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TUNEL 染色陽性を示すラット急性虚血心筋細胞の細胞膜透過性について
- TUNEL 染色法と Lanthanum イオンを用いた研究-

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[要旨]

虚血による心筋細胞死にはアポトーシスとネクローシスという二つの機序が存在する。DNA 断片化した急性虚血早期心筋細胞の細胞膜透過性が亢進しているか否かをはっきりさせる目的で本研究を行った。ラット左冠状動脈前下行枝を結紮し、心筋梗塞モデルを作製した。アポトーシスの検索には TUNEL 染色を、ネクローシスの検索には lanthanum イオン（La）に対して細胞膜透過性亢進を電子顕微鏡下で観察し判定基準に用いた。更に同一細胞での DNA 断片化と細胞膜透過性亢進を電子顕微鏡下で観察した。

虚血３０分後で、既に、26.7%の心筋細胞に、細胞膜透過性亢進が認められた。更に、虚血開始２時間後から DNA 断片化（TUNEL 陽性）と La に対する細胞膜透過性亢進が同一細胞で同時に出現する事が確認された。結論としては急性虚血による心筋細胞死の機序にはアポトーシスとネクローシスが同時に関与している。

KEY WORDS: Rat Myocardial Ischemia, TUNEL Method, Apoptosis, DNA Fragmentation, Lanthanum Ions.

[緒言・目的]

細胞死の機序にはアポトーシスとネクローシスの二通りがある。ネクローシスでは細胞全体もミトコンドリアも徐々に膨化し、細胞質の変化が先行する。最終的に細胞膜が破壊し、いわゆる細胞融解を起こす。これに対しアポトーシスでは、変化はまず核で始まり、核膜へのクロマチン凝集、核断片化が起き、細胞も核もまず縮小する。最終的にアポトーシス小体を形成し、マクロファージなどに貪食される。

虚血に陥った心筋が死に至る過程で、アポトーシスとネクローシスの機序がそれぞれ関与している事を示唆する所見が報告されるようになってきた。最近 Kajstura 等は、ラット心筋虚血実験において、虚血初期、即ち、虚血開始から 6 時間後までは虚血病巣形成にアポトーシスが主役を演じ，その後はネクローシスが主役になると報告し、アポトーシスがネクローシスに先行すると述べている。彼らの研究では、TUNEL 染色をアポトーシスの指標に用い、抗ミオシンモノクローナル抗体（以下 Ammab と略す）が、心筋細胞内に取り込まれること（膜破壊）をネクローシスの判定基準にしている。しかし、Ammab は分子量が大きく、膜の透過性亢進の指標には不適当だと思われる。一方、Koba 等は最近、Ammab よりはるかに分子量の小さい Lanthanum ($La(NO_3)_3$)（直径 40Å、以下 La と略す）を細胞膜透過性亢進の指標に用い、虚血心筋細胞について研究し、虚血開始 0.5 時間後において、既に、その 19%の心筋細胞に、La の取り込みが見られる事を報告している。

私どもは、ラット心筋梗塞モデルを用い、虚血早期（虚血開始 6 時間後まで）の虚血巣の心筋

細胞について、アポトーシスと細胞膜透過性の亢進（ネクローシス）が、同一の細胞で起き、アポトーシスが先行し、ネクローシスに移行するか、或いは、両者が別々の細胞で別々に進行するかをはっきりさせる目的で本研究を行った。アポトーシスの検索には、TUNEL 染色を、ネクローシスの検索には、La に対する細胞膜透過性亢進を電子顕微鏡下で観察し判定基準に用いた。

[材料及び方法]

心筋梗塞ラットの作成：Wistar 系ラット（6 週齢、雄)を用い、ネンブタール麻酔下，気管内挿管下にて第 3 肋間開胸後，冠状動脈前下行枝を結紮し、0.5,1,2,3,4,6 時間後に 3 匹ずつ屠殺。心虚血部位を切り出し、以下の処置を施し、検索した。対照群には、開胸術のみ施したラットを用いた。

1）虚血部位の組織片の一部を 20%緩衝ホルマリンで固定、パラフィン連続切片を作成し、H-E 染色による一般的病理組織検索、TUNEL 染色による DNA 断片化細胞の有無を検索した。また光顕下１０倍で梗塞巣の写真を撮って、200-300 個の細胞を数え、経時的に TUNEL 染色陽性細胞を定量化した。

2）残りの一部を電顕観察用に、小ブロックに細切し，1.3 % La を含む 2.6%グルタールアルデヒド固定液にて 2 時間固定し、La を含む洗浄液で洗った後、四酸化オスミウム（O_sO_4）で再固定、型通り樹脂包埋し超薄切片を作成した。電子染色を施した後、透過型電顕にて、各グループ 150 個ずつ La 陽性細胞検索し、陽性率を経時的に算出した。

3）上記２）の過程で、O_sO_4 で再固定する前に一部の細切ブロックから、8μm の厚さの凍結切片を作成し、TUNEL 染色を施し、光顕上で、TUNEL 陽性細胞を確認した後、そのままの状態で、即ち、スライドグラスに貼り付けたままの状態で、O_sO_4 で再固定し、樹脂包埋(pre-embedding 法)後、超薄切片を作成し、透過型電顕にて観察した。電顕上で TUNEL 陽性核を持つ心筋細胞を確認し、その細胞の細胞質に La が取り込まれているか否かを観察した。また、TUNEL 陰性細胞についても、La の細胞内取り込みを検索した。

[結果]

虚血巣の TUNEL 染色陽性出現率

虚血開始 30 分－４時間後の虚血巣では H&E 染色上組織学的変化は乏しいが、TUNEL 染色では虚血開始後 2 時間目から心内膜下に陽性細胞が認められ、虚血巣の TUNEL 陽性率は 17.9%±1.9 である。3、4 時間と時間の経過とともに陽性率が増加し、6 時間後では 54.9%±2.0 となった。

通常電顕による La 陽性細胞発現率

虚血開始 30 分後で既に、26.7%の心筋細胞が La を細胞質内に、限局性（特にミトコンドリアの外膜）に、取り込んでいた。3 時間後ではおよそ 81.5%の心筋細胞に、4,6 時間後では 90%以上の細胞内に La が沈着していた。一方対照群では、La は心筋細胞外にのみ認められ細胞内には認められなかった。

TUNEL 陽性細胞の La 透過性について

La 処理と TUNEL 染色の二重処理を施した虚血巣心筋細胞を検索した結果、虚血 2 時間後の群では、TUNEL 陽性細胞 10 個のうち 7 個が La 陽性、3 個が La 陰性であった。3 時間後の群では、TUNEL 陽性細胞 13 個のうち 10 個が La 陽性、3 個が La 陰性であった。また、TUNEL 陰性のうち、La 陽

性及びLa陰性細胞がそれぞれ少数ながら認められた。4−6時間後の群ではTUNEL陽性細胞、陰性細胞いずれにおいても、殆どの細胞にLaが細胞内に沈着していた。一方対照群では、細胞内のLa沈着は認められなかった。

以上の結果をまとめますと:

① 虚血後３０分で細胞膜透過性亢進が認められました。

② ２時間後にDNA断片化が検出され、DNA断片化と細胞膜透過性亢進が同時に起きている細胞が存在しました。DNA断片化のみ、または細胞膜透過性亢進のみが認められる細胞も少数ではあるが存在していました。

③ ４時間後以降,TUNEL陰性、陽性を問わず、殆どの細胞内にLaが認められました。

[考察]

本研究では、虚血30分後で、既に26.7%の心筋細胞に、細胞膜透過性亢進が認められた。更に、虚血２時間後からDNA断片化とLaに対する細胞膜透過性亢進が同一細胞で、同時に出現する事が確認された。これらTUNEL陽性で同時にLa陽性を示す細胞は、Wyllie等の言う典型的なアポトーシス細胞ではないことがはっきりした。しかし、これらの細胞群が虚血心筋の主要な細胞群である事もはっきりした。La陰性で、TUNEL陽性細胞が少数存在する。これらの細胞は典型的な意味でのアポトーシスに陥る細胞かもしれない。しかし、La陰性であることを電顕下で確定することは困難で、これらの点については今後の研究に待つ必要がある。虚血心筋では、La陽性細胞の一部に、TUNEL陰性の細胞が存在する。これらの細胞はいわゆる壊死細胞である可能性が高い。本研究の結果から判断すれば、Kajstura等の報告は、見かけ上アポトーシスがネクローシスに先行するという事を述べているのに過ぎないと言える。急性虚血による心筋細胞死の機序にはアポトーシスとネクローシスが同時に関与している。

[References]

(1) Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F: Features of apoptotic cells measured by flow cytometry Cytometry 1992;13: 795-808

(2) Gavrieli Y, Sherman Y and Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119:493-501.

(3) Gottlieb RA, Burieson KO, Kloner RA. Babior BM, Engier RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994; 94: 1621-1628.

(4) Hoffstein S, Gennaro DE, Fox AC, Hirsch J, Streuli F, Weissmann G: Colloidal lanthanum as a marker for impaired plasma membrane permeability in ischemic dog myocardium. Am J Pathol 1975; 79: 207-219.

(5) Itoh G, Tamura J, Suzuki M, Suzuki Y, Ikeda H, Koike M, Ito K: DNA fragmentation of human infarcted myocardial cells demonstrated by nick end labeling method and DNA agarose gel electrophoresis. Am J Pathol 1995; 146: 1325-1331.

(6) Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S,

Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independent, contributing variables of infarct size in rats. Lab Invest. 1995; 74: 86-107.
(7) Kajstura J, Mansukhani M, Cheng W, Reiss K, Krajewski S, Reed JC, Quaini F, Sonnenblick EH, Anversa P: Programmed cell death and the expression of the protooncogene Bcl-2 in myocyte during postnatal maturation of the heart. Exp Cell Res 1995; 219: 110-121.
(8) Kajstura J, Liu Y, Baldini A, Li B, Olivetti G, Leri A and Anversa P: Coronary artery constriction in rats: necrotic and apoptotic myocyte death. Am J Cardiol 1998;82:30k-41k.
(9) Kerr JFR, Wyllie AH and Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-257.
(10) Koba S, Konno S, Suzuki H and Katagiri T: Distribution of sarcolemmal integrity during ischemia and reperfusion of canine hearts as monitored by use of lanthanum ions and a specific probe. Basic Res Cardiol 1995; 90:203-210.
(11) Schmiedl A, Bach F, Fehrenbach H, Schnabel Ph.A and Richter J: Cellular distribution patterns of lanthanum and morphology of rat hearts exposed to different degrees of ischemic stress. Anat Rec 1995; 243:496-508.
(12) Selye H, Bajusz E, Grasso S and Mendel P: Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 1960; 11:398-407. (13) Tamura J: DNA fragmentation of rat infarcted myocardial cells demonstrated by TUNEL method and DNA agarose gel electrophoresis. J Aichi Med Univ Assoc 1996; 24: 493-502.
(14) Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Marumo P, Hiroe M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res 1994; 75: 426-433.
(15) Wyllie AH, Kerr JPR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 1980; 68: 251-306.
(16) Wyllie AH, Morris RG, Smith AL and Dunlop D: Chromatin cleavage in apoptosis: association with condenced chromatin morphology and dependence on macromalecular synthesis. J Pathol 1984; 142:67-77.

Rat Cardiomyocytes with TUNEL-Positive Nuclei Induced by Permanent Ischemia Show Increased Plasma-Membrane Permeability Monitored by a Combined Use of TUNEL Method and Lanthanum Ions

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Abstracts

The aim of this study was to clarify whether myocardial cells with DNA fragmentation induced by ischemia show increased plasma-membrane permeability (PMP), focusing on the early lesions of acute myocardial ischemia (AMI). Rat AMI was induced by a permanent occlusion of the left coronary artery. The TUNEL method was used for the demonstration of DNA fragmentation, and lanthanum ions (La) were employed for the increased PMP of cardiomyocytes. The increased PMP of cardiomyocytes having TUNEL-positive nuclei was determined by a combined use of the TUNEL method in frozen sections and electron microscopic identification of intracellular depositions of La.

Seven of 10 cardiomyocytes having TUNEL-positive nuclei revealed the deposition of La 2 hours after ischemia, and 10 of 13 showed La deposition 3 hours after ischemia. Almost all cardiomyocytes labeled with TUNEL stain showed intracellular deposition of La 4 and 6 hours after ischemia, respectively.

The evidence obtained from this experiment demonstrates that almost all cardiomyocytes labeled with TUNEL stain simultaneously show an increase of PMP in the AMI foci. In conclusion, the mechanism of myocardial cell death caused by permanent ischemia is due to a mixed form of apoptosis and necrosis.

Key words: rat myocardial ischemia, TUNEL method, apoptosis, DNA fragmentation, lanthanum ions.

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Apoptosis has been reported to occur after ischemia in cultured neonatal rat cardiomyocytes [14] and after ischemia/reperfusion in rabbit acute myocardial infarction, but never after ischemic injury alone [3]. However, we have reported that the nuclei of human infarcted myocardial cells with the morphological features of necrosis showed DNA fragmentation [5], and also that permanent ligation of the left coronary artery without reperfusion produced DNA fragmentation of rat myocardial cells at the ischemic foci [13]. Kajstura et al. [6] have reported that, in rat acute myocardial infarction, programmed cell death (apoptotic cell death) was the major independent form of cardiomyocyte cell death up to 4.5 - 6 hours after ischemia, and that necrotic cardiomyocyte cell death followed apoptosis. They also demonstrated recently [8] that coronary artery narrowing caused both necrotic and apoptotic cardiomyocyte cell death and that cardiomyocyte necrosis markedly exceeded apoptosis. In their experiment, as a tracer of the ruptured myocardial cell-membrane they used monoclonal anti-myosin antibody, which was relatively large in molecular size, and they reported that cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis) were a prominent aspect of myocardial damage only 6 hours after ischemia [6].

With regard to the PMP of myocardial cells injured by ischemia, a few studies have reported using lanthanum ions (La $(N0_3)_3$) (La) as a tracer of PMP [4, 11], since they had smaller molecules and have been successfully used as a tracer for the detection of increased permeability of the plasma membrane of cardiomyocytes [4, 11, 10]. Koba et al. [10] have reported that, when using La, increased PMP of myocardial cells occurred at the ischemic foci in the very early stage of ischemia. They detected increased PMP in 19%, and 30% of myocardial cells at 30 min, and at 1 hour after ischemia, respectively. Though Kajstura et al. [6] have

detected cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis), the tracer of necrosis they used was a monoclonal anti-myosin antibody which, as they pointed out, had a large molecular size, which was a limitation of the method. In this study, therefore, by using La as a tracer of PMP (necrosis) and the TUNEL method to monitor DNA strand breaks (apoptosis), we determined whether the PMP of rat myocardial cells at the ischemic foci is increased, focusing on the early stage of acute myocardial damage from 30 minutes to 6 hours after the induction of ischemia. We also tried to determine whether the membrane permeability of myocardial cells with DNA strand breaks (TUNEL-positive nuclei) increases by a combination of the TUNEL method and an electron microscopic identification of intracellular depositions of La.

Materials and Methods

Animals

Animals were cared for and maintained in accordance with the guidelines of the National Institute of Health (Bethesda Md. USA).

Male rats (Wistar strain, 6-weeks-old) purchased from Japan SLC, Inc. (Shizuoka, Japan) were used.

Experimental protocol

Acute myocardial infarction (AMI) was induced by the method described previously [13, 12]. In brief, after being anesthetized with an intraabdominal injection of sodium pentobarbital (35 mg/kg), male rats (120-130 g) were fitted with an endotracheal tube and placed on controlled respiration with positive pressure on inspiration. The chest was then opened through the third intercostal space. The heart was exteriorized quickly, and a 5-0 nylon ligature was placed under the visualized proximal segment of the left coronary artery (LCA). The ligature was then permanently ligated. Rats were then placed on controlled respiration until constant spontaneous respiration resumed. Those subjected to the same procedure but without coronary artery ligation were used as control animals.

Myocardial tissue preparation

Under ether anesthesia, each group of 3 rats was exsanguinated, and the hearts were removed at intervals of 30 minutes,1, 2, 3, 4 and 6 hours after ischemia. After the hearts were sliced horizontally, the ischemic portion was selected and cut into two slices, one of which was fixed with 10% neutral buffered formalin at room temperature (RM) for 24 hours, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin.

Identification of increased permeability of myocardial cell-membrane

The second slice was cut into small pieces for electron microscopic examination. After being fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La $(N0_3)_3$(La) [4], pH 7.4, for 2 hours, small pieces of the specimens were washed thoroughly with 0.1 M cacodylate buffered solution containing 1.3% La. After the pieces thus treated were postfixed for 2 hours with 1% osmium tetroxide in 0.1 M cacodylate buffered solution without added La, they were rinsed in PBS, dehydrated in alcohol, embedded in Epon 812, cut into ultrathin sections, and examined electron microscopically. The frozen sections (8 μm thick) of the remaining small pieces were stained with TUNEL method. The frozen sections stained with TUNEL were embedded in Epon 812 on the slide glasses for electron microscopic identification of La.

TUNEL method

DNA nick end labeling was performed to demonstrate DNA fragmentation. The staining method originally reported by Gavrieli et al. [2] was applied. In brief, after being deparaffinized and washed in double distilled water (DDW), the sections were incubated with 20 μm/ml proteinase K (PK) for 15 min at room temperature (RT), and then washed four times in DDW for 2 min. After the endogenous peroxidase was inactivated by covering sections with 2% H_2O_2 for 5 min at RT, the sections were rinsed in DDW, and immersed in TDT buffer (30 mM Tris-HCl buffer, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (0.3 e.u./ml) and biotinylated dUTP in TDT buffer were added to cover the sections, which were then incubated in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The sections were rinsed in DDW, covered with a 2% aqueous solution of BSA for 10 min at RT, rinsed again in DDW, and immersed in PBS for 5 min. Sections were then covered with streptavidin peroxidase, incubated for 10 min at 37°C, washed in DDW, immersed for 5 min in PBS, and stained with DAB (3,3'-diaminobenzidine tetrahydrochloride) for about 30 min at RT.

Quantitative analysis of TUNEL-positive cells and La-deposited cells

In brief, TUNEL-positive cells in three fields of infarcted areas were calculated in three experimental rats. The percentages of TUNEL-positive nuclei among the total number of nuclei at the ischemic foci were calculated at 0.5, 2, 3, 4 and 6 hours after the initiation of ischemia. At least 150 of both La-deposited and non-deposited cells were also counted under the electron microscope in each group. The percentage of La-deposited cells among total cells at the ischemic foci were also

calculated at 0.5, 2, 3, 4, and 6 hours after the induction of ischemia. Paired *t* tests were performed to detect significant differences.

Combination of TUNEL method and electron microscopic identification of intracellular deposition of La

In order to clarify whether or not the permeability of the plasma membrane of myocardial cells with TUNEL-positive nuclei increases, a combination study using the TUNEL method and electron microscopic identification of intra-myocardial cell depositions of La was done. In brief, small pieces of cardiac tissue fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La, pH 7.4, for 2 hours were cut into 8 micron-thick frozen sections and stained with the TUNEL method. After the TUNEL-positive cells was identified on frozen sections with a light microscope (Fig. 1), the frozen sections on slide glasses were then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffered solution without La, rinsed in PBS, dehydrated in alcohol, and embedded in Epon 812. Ultrathin sections were made and examined with a Hitachi 7000 electron microscope at 75 KV.

Results

Microscopic findings of hematoxylin and eosin (H&E) stain and TUNEL method

Thirty minutes to 4 hours after the induction of ischemia, no remarkable changes were observed in the ischemic area by H&E staining. However, by TUNEL staining, positive nuclei were identified in a limited number of myocardial cells in the ischemic subendocardial area 2 hours after the induction of ischemia. Six hours after ischemia, myocardial cells showed deep eosinophilia of the cytoplasm in H & E sections, while in TUNEL-stained sections most of the myocardial cell nuclei corresponding to those showing deep eosinophilic cytoplasm in H&E sections were positively stained.

A quantitative analysis of TUNEL-positive cells at ischemic foci

At the ischemic foci, 17.9% ± 1.9 of cells showed positive with TUNEL stain at 2 hours after ischemia. With

Figure 1. This shows a frozen section of the rat ischemic myocardial tissue which, after being fixed with 4% glutaraldehyde containing 1.3% La, was stained with the TUNEL method and embedded in Epon 812. Quadrangular area was examined by electron microscope. Arrow indicates TUNEL-positive nuclei. (x 25)

time TUNEL-positive nuclei increased thereafter, reaching 54.9% ± 2.0 at 6 hours after ischemia (Table 1).

Identification of La by electron microscope and quantitative analysis

Portions of myocardial tissues corresponding to those examined with the light microscope for both H & E and TUNEL stain were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffered solution containing 1.3% La, pH 7.4, for 2 hours, and then studied electron microscopically. Even 30 min after the onset of ischemia, 26.7% of myocardial cells in the ischemic area showed depositions of La. Percentages of La-deposited cells increased with time, reaching the more than 90% at 4 and 6 hours (Fig. 2a) after ischemia (Table 1). Percentages of La-deposited cardiomyocytes are significantly larger than those of TUNEL-positive nuclei in ischemic foci. In the control group, La was only visible in the intercellular space between two adjacent normal myocardial cells (Fig. 2b).

A quantitative analysis of PMP of myocardial cells with TUNEL-positive nuclei

The increased permeability of the plasma membrane of myocardial cells having TUNEL-positive nuclei at the ischemic foci was examined by electron microscope at 2, 3, 4 and 6 hours after the initiation of ischemia. Two hours after ischemia, 7 of 10 myocardial cells having TUNEL-positive nuclei revealed intracellular depositions of La (Fig. 3a), whereas the remainder did not. Three hours after ischemia, 10 of 13 myocardial cells having TUNEL-positive nuclei showed La depositions in myocardial cells (Fig. 3b), and the remainder did not. Almost all myocardial cells stained positive with the TUNEL method showed intracellular depositions of La at 4 and 6 hours after ischemia (Fig. 3c), respectively.

In control, intracellular depositions of La were not observed (Fig. 3d).

Discussion

It has been widely accepted that apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological and biochemical features [9, 15, 16]. Recent accumulating evidence indicates that the programmed cell death of cardiomyocytes plays an important role during the postnatal development of the heart [7] as well as during the occurrence of ischemia/reperfusion injury to the myocardium [3]. Furthermore, we previously reported that the nuclei of human infarcted myocardial cells having the morphological features of necro-

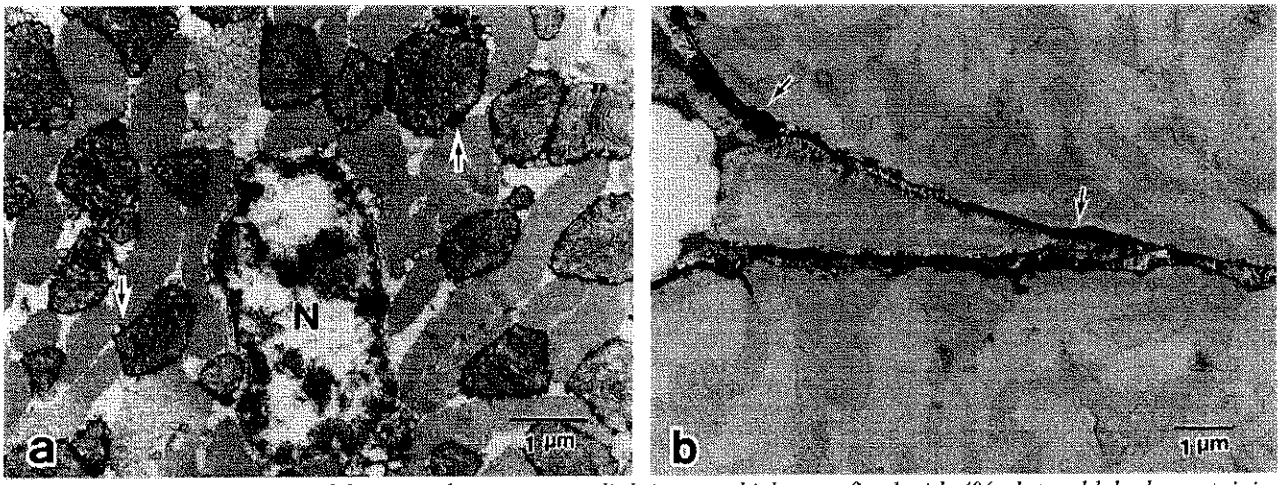

Figure 2. (a) Ultrastructure of the rat ischemic myocardial tissues which were fixed with 4% glutaraldehyde containing 1.3% La and postfixed with 1% osmic acid. Depositions of La (arrows) were found around the mitochondria. (Six hours after the onset of ischemia. This section was stained with uranyl acetate and lead citrate. N: nucleus). (b) Ultrastructure of the normal control rat myocardial tissues which were treated with the same procedures as the ischemic rat myocardial tissues. La were observed only in the intercellular space (arrows) at two adjacent normal myocardial cells. This section was stained with uranyl acetate and lead citrate.

Table 1. Frequency of lanthanum and TUNEL-positive cells at various time periods of ischemia.

	30 min (n = 1)	1 hr (n = 1)	2 hr (n = 3)	3 hr (n = 3)	4 hr (n = 3)	6 hr (n = 3)
TUNEL (%)			17.9 ± 1.9	26.2 ± 3.7	38.1 ± 2.1	54.9 ± 2.0
Lanthanum (%)	26.7	44.3	66.8 ± 5.0	81.5 ± 3.9	97.3 ± 2.8	99.5 ± 0.8

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. Lanthanum: Indicator of permeability of myocyte plasma membrane.

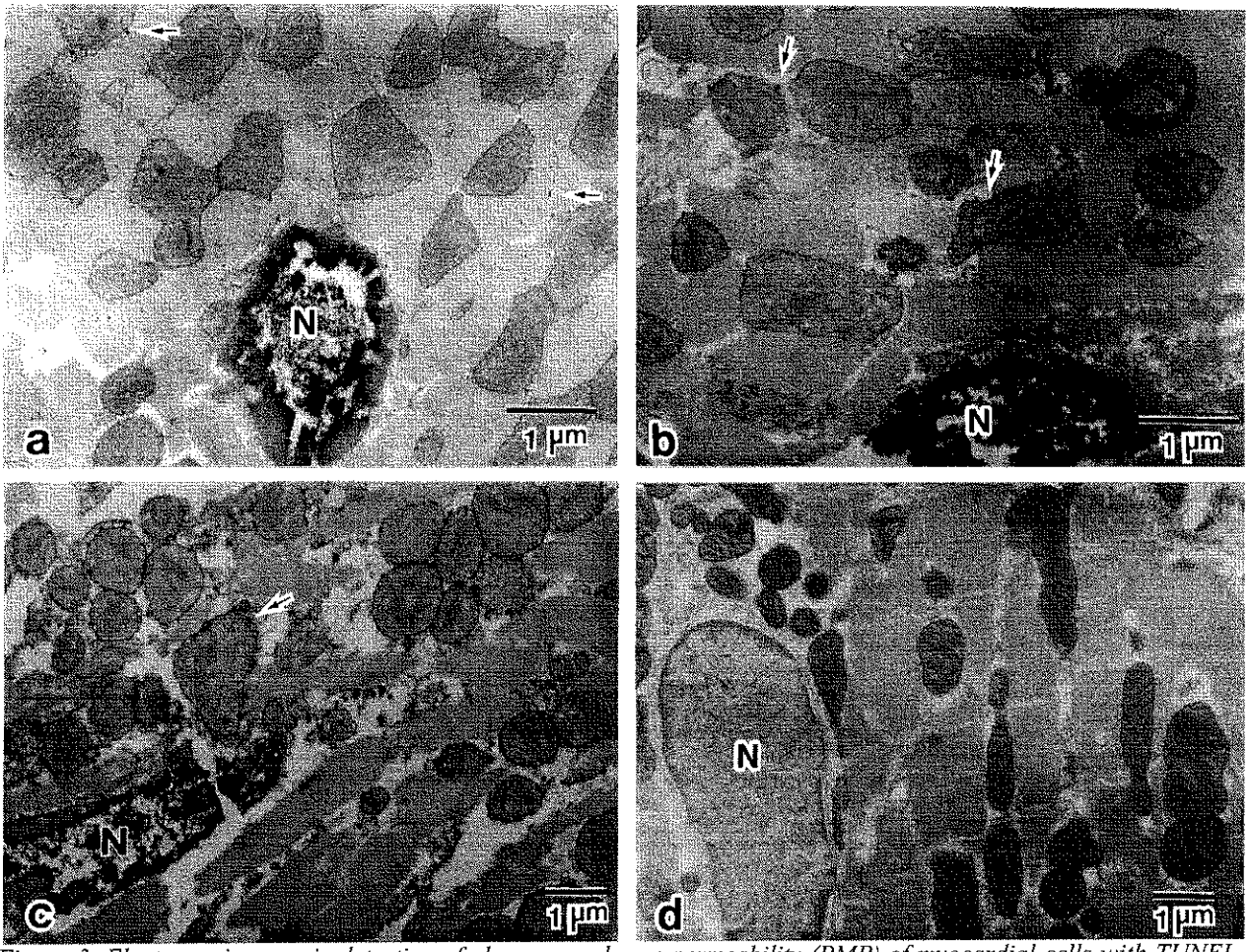

Figure 3. Electron microscopic detection of plasma-membrane permeability (PMP) of myocardial cells with TUNEL-positive nuclei. (a): 2, (b): 3 and (c): 6 hours after the onset of ischemia. Arrows indicate depositions of La which mainly located around mitochondria. (d): The normal control rat myocardial tissues which were treated with the same procedure as the ischemic rat myocardial tissues. No depositions of La were observed in the cytoplasm of myocardial cells. All sections were stained with lead citrate. N: nucleus.

sis (including both typical coagulation necrosis and contraction band necrosis) showed DNA fragmentation [5]. We also reported that permanent ligation of the left coronary artery without reperfusion produces DNA fragmentation of rat myocardial cells at the ischemic foci [13].

Kajstura et al. [6] reported that, by using both the TUNEL method for apoptosis and the anti-myosin antibody labeling method for necrosis, programmed myocardial cell death (apoptosis) appeared 2 hours after the induction of ischemia, that apoptosis was the major independent form of cardiomyocyte cell death up to 4.5-6 hours after coronary artery ligation, and that necrotic cardiomyocyte cell death follows apoptosis. Recently they [8] also reported that coronary artery narrowing caused both necrotic and apoptotic cardiomyocyte death. Necrosis is characterized by the loss of the plasma membrane integrity which remains intact in apoptotic cells [1]. Accordingly, if myocardial cell-injury caused by ischemia is due solely to the mechanism of apoptosis, the PMP of myocardial cells remains intact at an early phase of ischemia (2-6 hours after induction). In our study, the permeability of the plasma membrane was assessed by colloidal lanthanum, which has an average particle size of 40 Å, smaller than that of monoclonal anti-myosin antibody, and which penetrates spaces as small as 20 Å and has been successfully used as a marker for plasma membrane injury [4, 11, 10]. By using La as a tracer of PMP, we demonstrated that myocardial cells in the ischemic area showed increased permeability at a very early stage. The data obtained from our experiment were compatible with those by Koba et al. [10] who have reported that in ischemic rat hearts the deposition of lanthanum was found in 19% of ischemic cardiomyocytes after 30 min, and in 30% after 60 min of the initiation of ischemia. According to Kajstura et al. [6], cardiomyocytes showing both DNA strand breaks (apoptosis) and myosin labeling (necrosis) was a prominent mode of cell death only 6 hours after ischemia. However, in this experiment, we demonstrated that the PMP of almost all myocardial cells having TUNEL-positive nuclei showed increased plasma-

membrane permeability from 2 to 6 hours after ischemia, meaning that DNA strand breaks and increased PMP occurred simultaneously at identical myocardial cells in the ischemic foci. This finding was made possible by the use of La tracer used in this experiment, the molecular size of which is smaller than that of monoclonal anti-myosin antibody, and which penetrates spaces as small as 20 Å. In only a small number of the myocardial cells with TUNEL-positive nuclei was La deposition not detected. These cells, theoretically, might be pure apoptotic cells. However, some limitations exist in the use of the electron microscope for identifying the intracellular deposition of La, since some La-deposited cells might be regarded as La-non-deposited cells by electron microscopic examination, i.e., myocardial cells without deposition of La might not necessarily be true apoptotic cells.

The data obtained from our experiment indicate that almost all myocardial cells having TUNEL-positive nuclei simultaneously show an increase in PMP in the acute ischemic foci.

This strongly suggests that the mechanism of myocardial cell death induced by ischemia is due to two separate processes affecting identical myocardial cells in the ischemic foci, and that the mechanism of cell death is not due solely to either apoptosis or necrosis, but to a combination of both.

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References

[1] Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. *Cytometry* 1992; 13: 795-808.

[2] Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; 119: 493-501.

[3] Gottlieb RA, Burieson KO, Kloner RA, Babior BM, Engier RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 1994; 94: 1621-1628.

[4] Hoffstein S, Gennaro DE, Fox AC, Hirsch J, Streuli F, Weissmann G: Colloidal lanthanum as a marker for impaired plasma membrane permeability in ischemic dog myocardium. *Am J Pathol* 1975; 79: 207-219.

[5] Itoh G, Tamura J, Suzuki M, Suzuki Y, Ikeda H, Koike M, Ito K: DNA fragmentation of human infarcted myocardial cells demonstrated by nick end labeling method and DNA agarose gel electrophoresis. *Am J Pathol* 1995; 146: 1325-1331.

[6] Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independent, contributing variables of infarct size in rats. *Lab Invest* 1995; 74: 86-107.

[7] Kajstura J, Mansukhani M, Cheng W, Reiss K, Krajewski S, Reed JC, Quaini F, Sonnenblick EH, Anversa P: Programmed cell death and the expression of the protooncogene Bcl-2 in myocyte during postnatal maturation of the heart. *Exp Cell Res* 1995; 219: 110-121.

[8] Kajstura J, Liu Y, Baldini A, Li B, Olivetti G, Leri A, Anversa P: Coronary artery constriction in rats: necrotic and apoptotic myocyte death. *Am J Cardiol* 1998; 82: 30k-41k.

[9] Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239-257.

[10] Koba S, Konno S, Suzuki H, Katagiri T: Distribution of sarcolemmal integrity during ischemia and reperfusion of canine hearts as monitored by use of lanthanum ions and a specific probe. *Basic Res Cardiol* 1995; 90: 203-210.

[11] Schmiedl A, Bach F, Fehrenbach H, Schnabel Ph.A, Richter J: Cellular distribution patterns of lanthanum and morphology of rat hearts exposed to different degrees of ischemic stress. *Anat Rec* 1995; 243: 496-508.

[12] Selye H, Bajusz E, Grasso S, Mendel P: Simple techniques for the surgical occlusion of coronary vessels in the rat. *Angiology* 1960; 11: 398-407.

[13] Tamura J: DNA fragmentation of rat infarcted myocardial cells demonstrated by TUNEL method and DNA agarose gel electrophoresis. *J Aichi Med Univ Assoc* 1996; 24: 493-502.

[14] Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Marumo P, Hiroe M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994; 75: 426-433.

[15] Wyllie AH, Kerr JPR, Currie AR: Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; 68: 251-306.

[16] Wyllie AH, Morris RG, Smith AL, Dunlop D: Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* 1984; 142: 67-77.