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A Simple Method for Classification of Cell Death by Use of Thin Layer Collagen Gel for the Detection of Apoptosis and/or Necrosis after Cancer Chemotherapy

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To assess the efficacy of cancer chemotherapy, an important index is apoptosis of the target cells, which can usually be confirmed by electron microscopy (EM). We established a new experimental technique, whereby cancer cells (MKN45) were distributed in thin collagen gel as one or two cell layers, and cultured with anti-cancer drugs (5-FU and CDDP). The cells were stained with fluorescent Hoechst 33258 (Ho) and photographed, then with hematoxylin and eosin (H&E) and again photographed, and processed for EM. This approach allowed us to characterize the patterns of death of single cells in detail. There were six patterns of cell damage: two patterns of apoptosis, early peripheral condensation of chromatin and late apoptotic bodies, two patterns of necrosis, cytoplasmic swelling and washed-out images, and two further patterns, with morphological features of both apoptosis and necrosis, neither classified into necrosis nor apoptosis. The results show that cell death patterns can be mostly determined by combining observations of Ho and H&E-stained cells without the necessity for EM observation.

Key words: Collagen gel — Electron microscopy — Hoechst 33258 — Apoptosis and necrosis — Cancer chemotherapy

The objective of cancer chemotherapy is to kill cancer cells with as little effect as possible on normal cells. When necrosis is induced in cancer tissues by anticancer drugs, toxic chemical mediators from necrotic cells and/or inflammatory response will interfere with normal cellular physiology. Because of this, the induction of apoptosis, a specific type of cancer cell death has become a hot topic in recent studies of cancer chemotherapy, because it does not cause adverse effects.[1–4)]

Apoptosis is distinguishable from necrosis on the basis of morphological features under electron microscopy.[5)] These include nuclear fragmentation with condensed chromatin, condensation of cytoplasm, and membrane-bound apoptotic bodies. Necrosis, in contrast, is characterized by swelling of cytoplasm and mitochondria, rupture of the plasma membrane, and loss of chromatin.[6–10)] Recently, a new type of cell death, "aponecrosis" has been proposed,[11)] which has molecular and morphological features of both apoptosis and necrosis.

Diverse experimental methods have been adapted in order to identify apoptosis. Examples are fluorescence microscopy using DNA-specific staining reagents to detect coagulation of nuclear chromatin,[12)] agarose gel electrophoresis of DNA (DNA laddering)[13)] and *in situ* terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)[14)] which demonstrates the existence of DNA fragmentation, flow cytometry[15)] which measures DNA content, and hematoxylin and eosin (H&E) staining to assess the cell volume.

Among these methods, DNA laddering and the TUNEL assay are most commonly used for the estimation of apoptosis. It must be stressed, however, that a DNA ladder is not specific for apoptosis, because DNA fragmentation is also a feature of necrosis.[16–19)] Furthermore, 1) liver apoptosis triggered by transforming growth factor (TGF)-β1 is not accompanied by DNA fragmentation,[12)] 2) Jurkat cells, even after induction of apoptosis by anti-polimin, show lethal damage to cell membranes, but not DNA fragmentation/apoptotic bodies.[20)] The TUNEL assay is also prone to false positive or negative findings due to variation in prefixation time,[21)] extent of proteolysis and the reagent concentration in terminal deoxynucleotidyl transferase (TdT) assay.[22)]

Therefore classical electron microscopy is necessary for certain detection of apoptosis.[10, 18, 23)] However, this is highly technical, labor intensive and expensive, leading to difficulties in routine usage.[10)] Thus, a simple and reliable method for the estimation of apoptosis is required.

In this study we established a new experimental technique for assessment of single cells in tissue culture collagen gel by three methods: Hoechst 33258 (Ho) staining, H&E staining and electron microcopy (EM). The first two in combination proved sufficient to identify most cell death patterns without any necessity for EM.

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MATERIALS AND METHODS

Cell lines and culture medium The human gastric cancer cell line MKN45 was maintained in RPMI-1640, supplemented with 10% fetal calf serum and antibiotic/antimyotic solution (Sigma, St. Louis, MO), in 5% CO_2 and 95% air at 37°C. Cells in log phase were dispersed in culture medium by pipetting 20 times. This resulted in separation of cell colonies into single cells or clumps of 2–3 cells and these were used for the experiments.

Thin layer collagen gel preparation "Primaster" (Nitta Gelatin Inc., Osaka) was used for the collagen gel for tissue culture. In brief, 1250 μl of collagen solution was prepared according to the manufacturer's instructions and mixed with 125 μl of cell culture solution (1×10^6 cells/ml), and 1 μl of this mixture fluid was thinly painted using a micropipette tip onto sterilized "Lab-Tec" "Chamber Slides" (Nalge Nunc Int. Corp., Naperville, IL). The painted collagen solution was gelatinized at 37°C for 1 h under high humidity to avoid drying. Then additional culture medium (1 ml of RPMI-1640) was overlaid on each collagen gel. Cells were cultured in the thin layered collagen gel for 24 h and used for the experiment.

Drug effects The anti-tumor effects of 5-fluorouracil (5-FU, Kyowa, Tokyo) and cisplatin (CDDP) (Randa, Nihonkayaku, Tokyo) on MKN45 were examined after replacement of old culture medium with a mixture of 5-FU (100 μg/ml) and Randa (20 μg/ml) in RPMI-1640, and cultured at 37°C.

Sampling Specimens were processed for morphological examination at 0, 3, 6, 12, 24 h after the drug exposure for induction of apoptosis. Cells were fixed with 2.5% glutaraldehyde in 0.1 *M* phosphate buffer.

Nuclear staining and fluorescence microscopy After three rinses with 0.1 *M* phosphate buffer (pH 7.4), cells in the gel were stained with 1 μM Ho for 15 min, washed, and observed under a non-confocal fluorescence microscope with excitation at 360 nm. All cells were photographed.

Light microscopy After staining with Ho, cells in the gel were stained with hematoxylin for 15 min, washed with water, and then stained with eosin for 5 min (H&E). Then the specimens were observed under a light microscope and photographed.

Electron microscopy After H&E staining, cells in gels were fixed in half-strength Karnovsky fixative (a mixture of paraformaldehyde and glutaraldehyde). After three

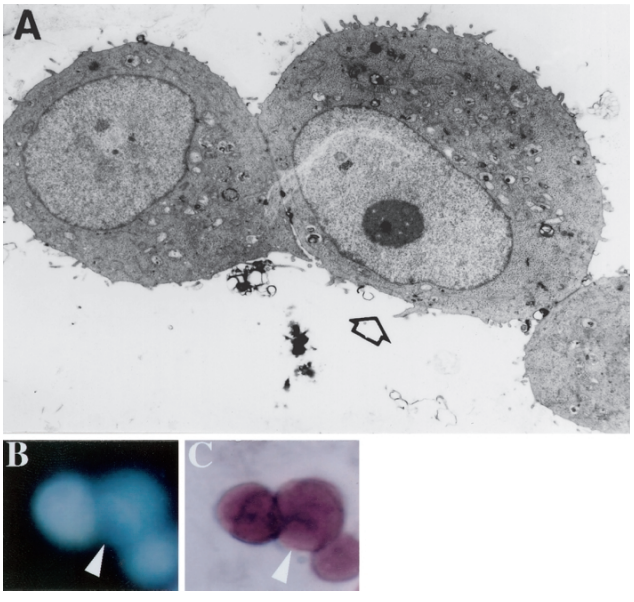

Fig. 1. (A) Control cell, before exposure to the anti-cancer drugs 5-FU and CDDP, exhibits normal ultrastructural features under EM (open arrow) (×2500). (B) On Ho staining, the nucleus is blue (white arrow), and (C) on H&E staining, the cell shows good integrity (white arrow).

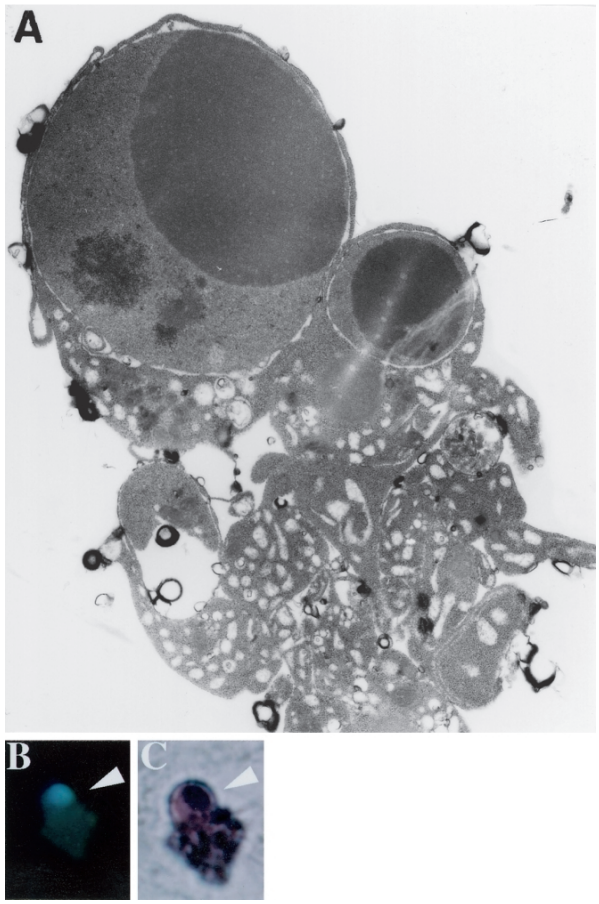

Fig. 2. (A) Apoptotic bodies with nuclear condensation under the EM (×4000). (B) The same region of condensed chromatin shows deep blue Ho staining (white arrow), and (C) is stained strongly with hematoxylin (white arrow).

rinses with phosphate buffer, the specimens were postfixed with 1% OsO_4 for 2 h at 4°C, dehydrated through ascending concentrations of alcohol (50, 70, 80, 90, and 100%) in propylene oxide for 15 min, and embedded in Epon 812. Polymerization was performed for 3 days at 60°C and the embedded specimens were detached from slides by heating. Ultrathin sections 80–100 nm in thickness were cut with a diamond knife, stained with uranyl acetate, and observed under an electron microscope (IEM-1010, Nippon Denshi, Tokyo).

Identification of cells The main purpose of this study was to compare the results of three modes of observation of the same cells. Towards this purpose, cells with good appearance in electron micrographs were chosen for comparison. Based on morphology and location within the sample, the same cell was then identified in the Ho and H&E staining photos.

RESULTS

MKN45 cells were successfully cultured in our thin-layer gel. Cells grown in one or two layers were evenly distributed in thin layers, allowing the whole morphology of each cell to be easily observed and photographed under the light microscope.

Control Control cells, before exposure to anti-cancer drugs, exhibited normal ultrastructural features under EM (open arrow of Fig. 1A). They were round in shape and equipped with well developed microvilli on the cell surface. The nucleus had a round nucleolus and dispersed chromatin. The integrity of the plasma membrane, mitochondria and other cytoplasmic structures was well maintained. The Ho staining profile of the same cells was examined retrospectively. The nucleus exhibited a round shape and stained blue (white arrow of Fig. 1B). On H&E staining, the same cell had a round-shaped nucleus and a basophilic nucleolus. The cytoplasm was eosinophilic (white arrow of Fig. 1C). Thus, control cells with good morphology by H&E staining had good ultrastructural integrity under EM.

Cells treated with anti-cancer drugs When cells were treated with 5-FU and CDDP, most exhibited morphological changes, which could be classified into six groups based on electron microscopic observations.

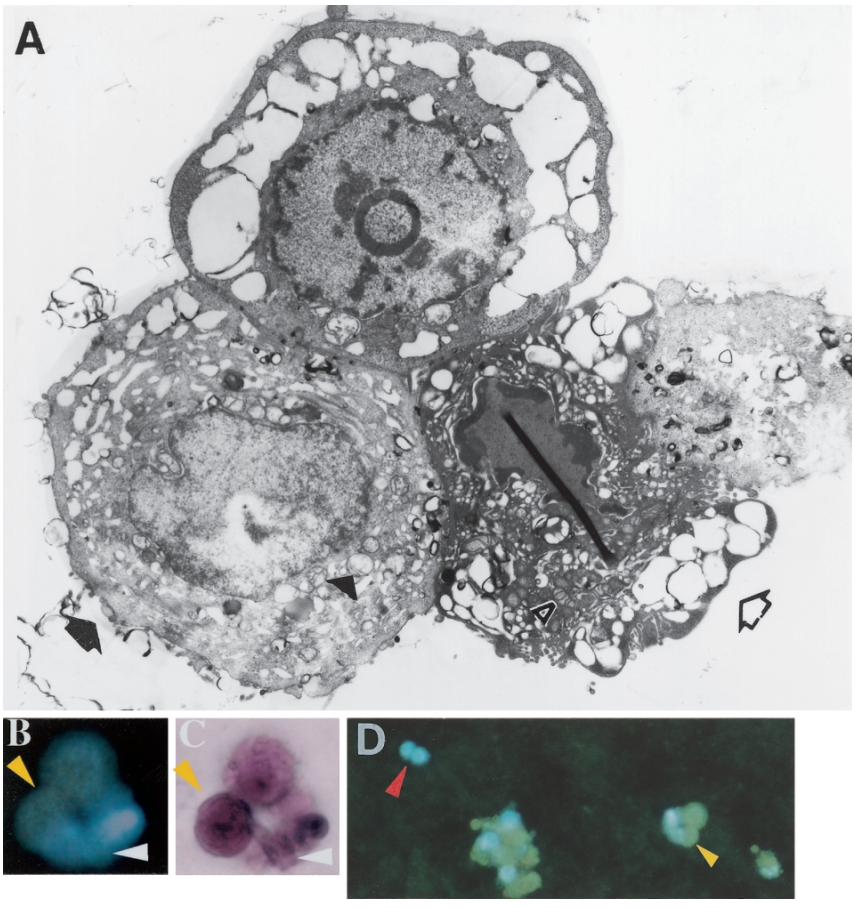

Fig. 3. (A) Peripheral condensation and dark cytoplasm under the EM (open arrow) with mitochondria maintaining good morphology (open triangle) (×2500). (B) Peripheral nuclear condensation is not confirmed by Ho staining (white arrow), but (C) H&E staining reveals such condensation by deep basophilic staining (white arrow). (A) Cytoplasmic vacuoles and swollen mitochondria (closed triangle) in another cell (closed arrow) under the EM (×2500). (B) On Ho staining, the same cell is not blue (yellow arrow), (C) while on H&E staining there is no salient difference from control cells (yellow arrow). (D) A low-magnification photograph of Ho staining after 48 h drug exposure. At this time point, many cells are not blue including the fourth group cell (yellow arrow), whereas the normal cell (red arrow) is blue, acting as built-in control of Ho staining specificity.

The first group was characterized by apoptotic bodies with nuclear condensation (Fig. 2A). The nucleus was round in shape, filled with condensed chromatin and surrounded by remnants of cytoplasm. The cytoplasm was condensed, fragmented and contained numerous vacuoles. The integrity of the plasma membrane was preserved, but without microvilli. These features are in good agreement with the classical criteria for apoptosis. The region of condensed chromatin was deep blue on Ho staining, whereas the fragmented cytoplasm was not stained (white arrow of Fig. 2B). On H&E staining, the former was strongly basophilic whereas the latter was eosinophilic and contained some basophilic granules. The integrity of the nucleus and cytoplasm was well maintained (white arrow of Fig. 2C).

The second group was characterized by peripheral nuclear condensation. Under the EM, cells of this type were compactly atrophied in isolation and showed shrinkage of the nucleus accompanied by peripheral nuclear condensation of chromatin, and dark cytoplasm filled with extensive vacuoles (open arrow of Fig. 3A). Cellular organelles such as mitochondria maintained good morphology (open triangle of Fig. 3A), consistent with apoptosis before proceeding to an apoptotic body. Peripheral nuclear condensation was not confirmed by Ho staining (white arrow of Fig. 3B), but it was revealed by deep basophilic H&E staining (white arrow of Fig. 3C).

The third group was characterized by washed-out images of cytoplasm and nucleus with loss of integrity of plasma membrane and dispersed contents. The nuclear membrane was ruptured, chromatin was dispersed, and mitochondria were severely swollen, in good agreement with the classical criteria for end stage necrosis (open arrow of Fig. 4A). On Ho staining, the cell was not blue, but its round shape was identifiable by green autofluorescence (white arrow of Fig. 4B). On H&E staining, the cell showed dispersed basophilic spots within weakly eosinophilic cytoplasm (white arrow of Fig. 4C).

The fourth group was characterized by cytoplasmic vacuoles (closed arrow of Fig. 3A). This cell had some damage to the cytoplasm, including multiple vacuoles and swollen mitochondria (closed triangle of Fig. 3A). However, the nucleus was nearly intact and plasma membrane

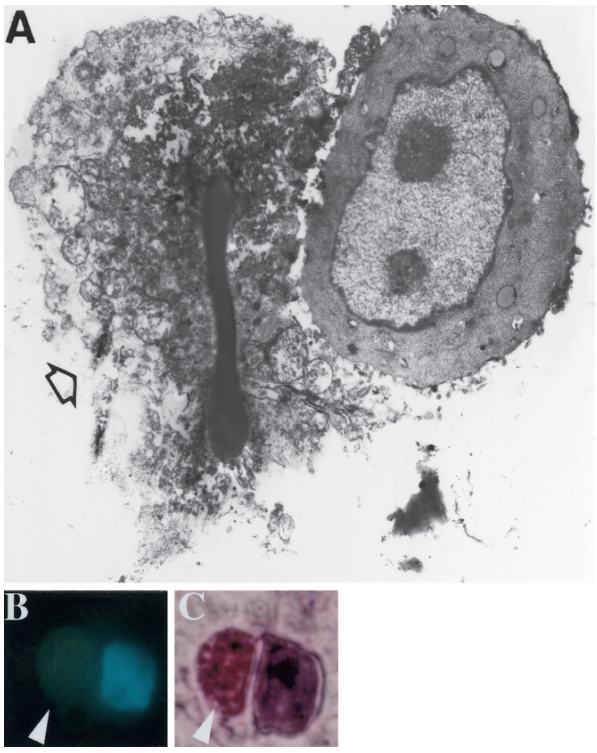

Fig. 4. (A) Washed-out image of cytoplasm and nucleus under the EM (open arrow) (×2500). (B) The cell lacks blue coloration on H&E staining (white arrow). (C) On H&E staining, the cell shows dispersed basophilic spots within weakly eosinophilic cytoplasm (white arrow).

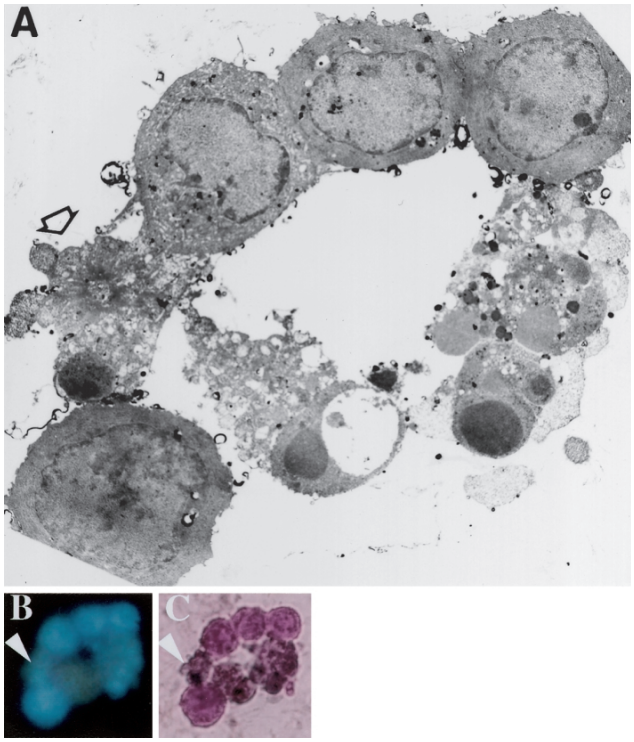

Fig. 5. (A) A cell with morphological features of both apoptosis (nuclear condensation) and necrosis (washed-out cytoplasm) under the EM (open arrow) (×1500). (B) Pale blue appearance on Ho staining (white arrow). (C) On H&E staining, the same region of nuclear condensation is stained strongly basophilic, and cytoplasm has basophilic spots within weakly eosinophilic cytoplasm (white arrow).

integrity was maintained. The microvilli on the cell surface were less developed than with the control cells, in line with the classical criteria for early stage necrosis. On Ho staining, the cell was not blue (yellow arrow of Fig. 3B). H&E staining clearly revealed the shape of the cell. The nucleus was basophilic and the cytoplasm was eosinophilic. Thus, there was no clear difference from control cells (yellow arrow of Fig. 3C). Fig. 3D is a low magnification photograph of Ho staining after 48 h drug exposure. At this time point, many cells were not blue, including early necrotic cell (yellow arrow), whereas a normal cell (red arrow) was blue, acting as a built-in control of Ho staining specificity.

The fifth group had morphological features of both apoptosis and necrosis (open arrow of Fig. 5A). This cell was shrunk in isolation and showed round-shaped nuclear condensation of chromatin (characteristic of apoptosis), whereas mitochondria were swollen and the cytoplasm was washed-out (characteristic of necrosis). On Ho staining the cell was pale blue (white arrow of Fig. 5B), but on H&E staining, the region of nuclear condensation under the EM was stained strongly basophilic. Several basophilic spots were apparent within weakly eosinophilic cytoplasm (white arrow of Fig. 5C).

The sixth group had morphological features not falling into any known cell death type. The illustrated cell was swollen due to giant vacuoles in the cytoplasm, pushing the other organelles towards the cell periphery and the perinuclear region, the cell surface being devoid of microvilli (open arrow of Fig. 6A). On Ho staining, this cell was not blue (white arrow of Fig. 6B). On H&E staining, the shape of the cell and the nucleus were clearly identifiable and the periphery of the nucleus was strongly stained with hematoxylin, whose staining was more conspicuous in comparison with that of normal cells. Giant vacuoles were devoid of staining (white arrow of Fig. 6C).

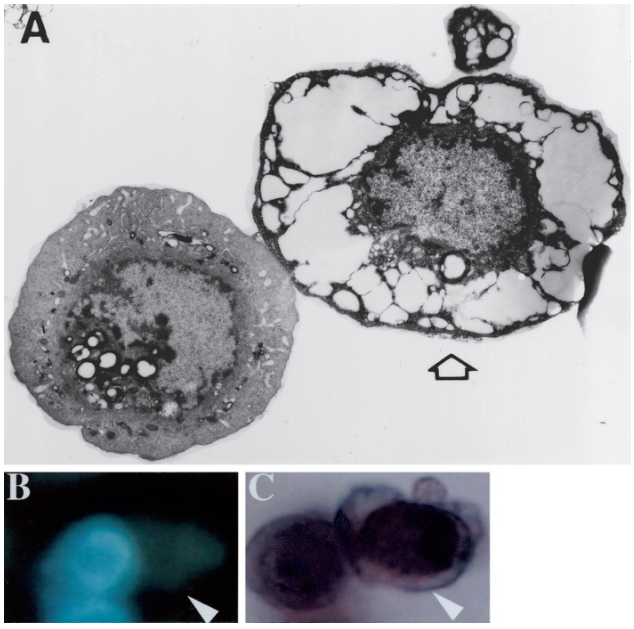

Fig. 6. (A) A cell with cytoplasm occupied by giant vacuoles under the EM (open arrow) (×2000), (B) lacks blue on Ho staining (white arrow), and (C) exhibits atypical morphological features on H&E staining (white arrow).

DISCUSSION

According to current concepts, cell death is classified into apoptosis or necrosis on the basis of morphological observation with EM considered as the gold standard for detection. In this study, we observed the effects of anticancer drugs on MKN45 cells by EM and identified six patterns of cell damage: two patterns of apoptosis, early peripheral condensation of chromatin and late apoptotic bodies, two patterns of necrosis, cytoplasmic swelling and

Table I. Characterization of Six Patterns of Cell Death Classified with the Three Observation Methods

	Control	Apoptosis		Necrosis		Aponecrosis	Unclassified
		Early	Late	Early	Late		
EM	Good integrity	Peripheral nuclear condensation	Apoptotic bodies	Cytoplasmic vacuole	Washed-out image of cytoplasm	Nuclear condensation and washed-out image of cytoplasm	Giant vacuoles in cytoplasm
Hoechst 33258	(+)	(±)	(++)	(−)	(−)	(±)	(−)
H&E Hematoxylin	(+)	(+)	(++)	(+)[a)]	(+)[c)]	(+)	(+)
Eosin	(+)	(±)	(+)	(+)[b)]	(+)[d)]	(±)	(−)

EM, electron microscopy; H&E, hematoxylin and eosin stain.
a) Round basophilic nucleus.
b) Eosinophilic cytoplasm.
c) Dispersed basophilic spot.
d) Weakly eosinophilic cytoplasm.

washed-out images, and two further patterns, with morphological features of both apoptosis and necrosis, neither classified into necrosis nor apoptosis. Combined observation of Ho and H&E-stained preparations allowed identification of normal cells, apoptosis and necrosis, and furthermore, subclassification. The results of our new technique show that identification of most cell death patterns is possible by combining observations of Ho and H&E staining without EM (Table I).

It is known that there is an intermediate type of cell death, that is, aponecrosis induced by incomplete execution of the apoptotic program due to intracellular ATP reduction.[11] In fact, we found cells with the features of both apoptosis and necrosis. These may be secondary changes after DNA damage and growth imbalance, which may result in alteration of the morphology of cell death.[15] Our results revealed that the combination of Ho and H&E staining can distinguish most cell death patterns, but is unable to distinguish aponecrosis and early apoptotic cells.

Our new approach for examination of cell death patterns in culture gels using light and fluorescence microscopy, has many advantages. 1) It is rapid, and the whole procedure for Ho and H&E staining can be accomplished in 1 h. 2) The experimental methods are very simple and easy. Comparison of images is possible at equal magnification with a motorized, computer-controlled microscope. 3) There are few artifacts due to initial fixation because physical loads such as suspension or centrifugation, usually needed in conventional techniques, are not employed. Therefore the morphology has fewer artifacts and should accurately reflect the natural condition. 4) Further staining is possible, including immunohistochemical staining and TUNEL. 5) The use of collagen gel as an extracellular matrix mimics the condition *in vivo*,[24–26] except in the case of small cell lung cancer cells that become resistant to chemotherapeutic agents when they adhere to an extracellular matrix.[27]

In cancer chemotherapy, induction of apoptosis in cancer tissue without affecting normal cells is a major objective, so a simple and reproducible method is required to identify apoptosis in experimental studies of cancer chemotherapy. Our new method appears to meet the requirements.

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